UNIVERSITY OF ALBERTA

REGULATION OF NATURAL KILLER CELL CYTOTOXIC ACTIVITY BY CLASS I MHC

BY

CHEW SHUN CHANG ©

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of the requirement for the degree of **DOCTOR OF PHILOSOPHY**

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ABSTRACT

Natural killer (NK) cells are a population of lymphocytes which exhibit high cytotoxic activity against transformed and virally infected cells. Although NK cells do not express T cell receptors, they do express class I Major histocompatibility complex-specific regulatory receptors belonging to the Ig superfamily and the animal C-type lectin superfamily. In mice, NK cell cytotoxic activity is regulated by animal C-type lectin-like receptors such as CD94/NKG2 and Ly-49.

To study the interaction of the murine Ly-49A NK cell inhibitory receptor and D^d, *in vitro* cell adhesion assays were used. Profound changes in D^d-expressing cells binding to isolated Ly-49A was observed over a narrow range of Ly-49A density, demonstrating that engagement of D^d is greatly influenced by Ly-49 density. Since Ly-49A can bind sulfated carbohydrates, the possibility that D^d can be sulfated was examined. Sulfated D^d was detected in activated T cells and in different tumor cell lines, and the sulfate modification was found to be added on mature *N*-linked carbohydrates. Using the cell adhesion assays, the data indicated D^d lacking sulfated carbohydrates is unable to interact with Ly-49A, especially when Ly-49A density is limiting. With these data, a coordinate interaction model for Ly-49A-D^d interaction is proposed.

To facilitate the study of Ly-49 receptor functions, two new mAbs specific for Ly-49G2^{B6} were generated, one of which exhibits no cross reactivity to other known B6 Ly-49 family members. Using these reagents, we demonstrated that expression of D^d can indeed inhibit Ly-49G2$^+$ NK cell function. In addition, Ly-49G2 expression is reduced with the introduction of a 129/J genetic background, suggesting that nonclassical class I MHC may be the major ligand for Ly-49G2.

The ability of mCD1.1, a nonclassical class I MHC, to regulate NK cell cytotoxic activity was also investigated. The results indicate that mCD1.1 expression can protect target cells from lysis by a small population of NK cells, and these NK cells can be

expanded by culturing with IL-2. The data further suggest the existence of a conserved mCD1.1-specific inhibitory receptor(s).

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ABBREVIATIONS

• Ab	Antibody
• Ag	Antigen
• A-LAK	Adherent lymphokine-activated killer
• ATCC	American Type Culture Collection
• β_2m	Beta-2-microglobulin
• BCS	Bovine calf serum
• BSA	Bovine serum albumin
• cDNA	Complementary DNA
• Con A	Concanavalin A
• CRD	Carbohydrate recognition domain
• CTL	Cytotoxic T lymphocyte
• DMEM	Dulbecco's modified Eagle's medium
• DOC	Deoxycholate
• ECL	Enhanced chemiluminescence
• ELISA	Enzyme-linked immunosorbent assay
• Endo F	Endoglycosidase F
• Endo H	Endoglycosidase H
• ER	Endoplasmic reticulum
• E:T	Effector-to-target cell ratio
• FACS	Fluorescence activated cell sorter
• FCS	Fetal calf serum
• FITC	Fluorescein isothiocyanate
• h	Hour
• H-2	Mouse major histocompatibility complex
• Hepes	*N*-2-hydroxyethylpiperazine-*N'*-2-ethansulfonic acid
• HLA	Human leukocyte antigen
• HRP	Horseradish peroxidase

• Ig	Immunoglobulin
• IL	Interleukin
• i.p.	Intraperitoneal
• ITAM	Immunoreceptor tyrosine-base activation motif
• ITIM	Immunoreceptor tyrosine-base inhibitory motif
• i.v.	Intravenous
• kDa	Kilodalton
• mAb	Monoclonal antibody
• 2-ME	2-β-Mercaptoethanol
• min	Minute
• MFI	Mean fluorescence intensity
• MHC	Major histocompatibility complex
• M_r	Relative molecular mass
• NA-LAK	Nonadherent lymphokine-activated killer
• NK	Natural killer
• NKC	NK gene complex
• NP	Influenza nucleoprotein
• NP-40	Nonidet-P40
• NWNA	Nylon wool nonadherent
• OD	Optical density
• PAGE	Polyacrylamide gel electrophoresis
• PBS	Phosphate buffered saline
• PMSF	Phenylmethylsulfonyl fluoride
• Poly I:C	Polyinosinic-polycytidylic acid
• RBC	Red blood cell
• rpm	Rotation per minute
• SD	Standard deviation
• SDS	Sodium dodecyl sulfate
• TAP	Transporter associated with antigen processing

• TCR	T cell receptor
• U	Unit

CHAPTER I

GENERAL INTRODUCTION

A. Self and Non-Self Discrimination by The Immune System

One of the distinctive features of the immune system is its ability to discriminate self from non-self. Under normal physiological conditions, an intact immune system will not respond to self-antigens (Ags) and therefore be self-destructive. In other words, a state of self-tolerance has to be induced and maintained. This characteristic has been the major driving force that shapes most concepts in immunology. The self and non-self element of immune recognition, for instance, is heavily integrated in the "clonal selection" theory which provides an explanation as to how the immune system selectively expand lymphocyte populations that recognize foreign antigens (Ada and Nossal. 1987).

How does the immune system determine whether an antigen is a self-antigen or foreign in origin? What are the underlying mechanisms that govern immunological self-tolerance? There are no single and easy answers to these questions. Nevertheless, some of the fundamental aspects of these central immunological issues have been addressed over the years. For instance, B lymphocytes secrete immunoglobulins (Igs) which bind to and neutralize foreign antigens, whereas T lymphocytes achieve their antigen specificity by expressing T cell receptors (TCRs). To maintain self-tolerance, potential self-reactive B and T lymphocytes are eliminated through clonal deletion and clonal anergy. Since B and T lymphocytes with new antigen specificity are constantly being generated, self-tolerization therefore has to be an ongoing process (Nossal. 1983). Furthermore, since not all self-antigens are available to both B and T lymphocytes during their maturation in the bone marrow and thymus, respectively, some sort of peripheral tolerization mechanism is also expected to take place to ensure these self-reactive lymphocytes are removed or anergized (Roser. 1989).

In addition to B and T cells, natural killer (NK) cells are also capable of discriminating self from non-self, although they do not express antigen specific receptors found on B and T cells. The molecular basis of the NK cell regulation serves as the focus of this thesis.

B. The Major Histocompatibility Complex

Historical Note

The discovery of the major histocompatibility complex (MHC) antigens was essentially sprung from the studies of blood group genetics and tumor transplantation. In 1936, using rabbit serum raised against mouse blood cells, Gorer was able to correlate the ability of the host to reject the transplanted tumor depending on whether or not they shared an antigen that he termed "antigen II" (Klein. 1986). This antigen II was shown to be distinct from the known ABO blood groups at the time. Subsequently, through the generation and analysis of congenic mice, it was demonstrated that the "determining factors" which are involved in rejection of the transplanted tumor is indeed antigen II (Klein. 1986). The determining factors were eventually referred to as histocompatibility genes and the first gene isolated was designated as H-2 (the "2" simply refers to antigen II). These gene products were later shown to be the major factors controlling skin graft rejection. Because further studies demonstrated that these genes are located in a single region of chromosome 17, they are now referred to as the major histocompatibility complex. Similar genetic loci are also found on human chromosome 6, and are referred to as the human leukocyte antigens (HLAs) (Klein. 1986).

Since the discovery of the MHC, genes encoded in these loci have been extensively studied. In addition to rejection of tumors and skin grafts, MHC gene products are known to play a central role in organ transplantation and self and non-self discrimination. The MHC consists of multiple genes which are involved in different aspects of the immune system. For instance, these genes include those coding the class I and II MHC molecules. Class I MHC molecules present peptide derived from endogenous antigens to the CD8$^+$ T lymphocytes or cytotoxic T lymphocytes (CTLs) (York and Rock. 1996). In contrast, class II MHC molecules are involved in presenting peptides derived from exogenous antigens to CD4$^+$ T lymphocytes or T helper (Th) (Braciale et al., 1987). In addition to these antigen presenting molecules, genes coding for other components of the class I and class II antigen processing and presentation pathways, and of the complement pathway, are also located within the MHC. As this thesis is focused on NK cell functions regulated by class I MHC molecules, only class I antigen presenting molecules will be discussed in the following sections.

Classical Class I MHC Molecules

Class I MHC molecules are expressed by almost all nucleated cells. They are expressed on the cell surface as two non-covalently linked polypeptides. In the murine system, the larger component or the heavy chain is a 44 to 47 kDa transmembrane glycoprotein encoded by the K, D and L loci, whereas in human, they are encoded by the HLA-A, B and C loci. Class I heavy chains, in turn, non-covalently associate with the 12 kDa β-2-microglobulin (β_2m), commonly referred to as the light chain, which is encoded outside the MHC (Klein. 1986; Robinson and Kindt. 1989). Association with the light chain is important in class I MHC molecule intracellular trafficking and cell surface expression. In cells deficient in β_2m expression, few class I MHC molecules are detected on the cell surface (Koller et al., 1990).

A distinctive characteristic of class I MHC molecules is their extreme polymorphism. For instance, more than 50 alleles have been identified in the murine K locus. Similar extreme polymorphism is also observed in other murine MHC loci and those of other species. Due to their polymorphism, class I MHC molecules encoded by the murine K, D, L loci and human HLA-A, B and C loci are referred to as classical class I

MHC molecules. To facilitate the study of these MHC encoded molecules, the combination of the alleles of each locus (including class II MHC loci) on a chromosome is operationally defined as a MHC haplotype. For example, the haplotype of the inbred homozygous mice Balb/c mouse strain is designated as H-2^d and the classical class I MHC encoded in the D and K loci are referred to as D^d and K^d, respectively.

Even with their extreme polymorphism, all classical class I MHC molecules adopt a similar three-dimensional structure. From the crystal structure, the extracellular portion of class I MHC molecules is organized into three distinct domains; the $\alpha 1$, $\alpha 2$ and $\alpha 3$ domains (Bjorkman et al., 1987). The $\alpha 1$ and $\alpha 2$ helices fold coordinately to comprise the peptide binding groove of the antigen presenting molecule, whereas the $\alpha 3$ domain is where the majority of contacts with β_2m take place (Bjorkman et al., 1987). The polymorphism of classical class I MHC molecules tends to concentrate in the $\alpha 1$ and $\alpha 2$ domains (Watts et al., 1989). These polymorphisms are particularly concentrated in the peptide binding groove and at the top of the two alpha helices pointing toward the TCR. This accounts for the large repertoire of peptides that are bound by class I MHC molecules collectively. Also, since the TCR interacts with the $\alpha 1$ and $\alpha 2$ alpha helices and the bound peptide (Garcia et al., 1996), MHC polymorphism underpins MHC-restriction and self and non-self discrimination by $CD8^+$ T cells (Zinkernagel and Doherty. 1974).

As for post-translational modification, all of the murine classical class I MHC molecules possess two conserved *N*-linked glycosylation sites, N86 and N176, residing in the $\alpha 1$ and $\alpha 2$ domains, respectively (Watts et al., 1989). A third glycosylation site in the $\alpha 3$ domain is also found in some class I MHC alleles such as D^b, K^d and L^d (Watts et al., 1989). The *N*-linked glycans of class I MHC are involved in its interaction with calnexin in the endoplasmic reticulum (ER) which facilitates class I MHC assembly and maturation (Degen and Williams. 1991; Degen et al., 1992). Thus, it is not surprising that removal of the class I MHC *N*-linked glycosylation sites results in reduction of class I MHC cell surface expression (Shiroishi et al., 1985; Barbosa et al., 1987). However, no other functional roles have been definitively assigned to this post-translational modification on class I MHC molecules, as the *N*-linked carbohydrates have been shown to be not involved in class I MHC interaction with TCR (Goldstein and Mescher. 1985; Miyazaki et al., 1986).

Nonclassical Class I MHC Molecules

In addition to the classical class I MHC molecules, both human and mouse also carry genes encoding for other antigen presenting molecules in the MHC loci. Amino acid sequence analysis shows that they share identity to classical class I MHC molecules. However, in sharp contrast to classical class I MHC molecules, they are generally non-polymorphic (Shawar et al., 1994). To distinguish them from the classical class I MHC, they are referred to as nonclassical class I MHC molecules. It is noteworthy that these nonclassical class I MHC molecules still adopt the classical class I MHC folding, with clearly defined $\alpha1$, $\alpha2$ and $\alpha3$ domains and they are associated with β_2m (O'Callaghan et al., 1998). In the murine system, they are encoded by the H-2Q, H-2T and H-2M loci, whereas HLA-E to -J are the loci at which human nonclassical class I MHC molecules are encoded (Shawar et al., 1994). To add to the complexity of the system, multiple genes can be encoded at each locus. For instance, at least 10 genes have been identified in the murine H-2Q and H-2T regions (Shawar et al., 1994; Soloski et al., 1995). It is important to note that some of the genes that reside at these loci are pseudogenes and deemed to be non-functional. Nevertheless, most of them do have intact open reading frames and at least some of them have been demonstrated to present antigens to T cells (Vidovic et al., 1989; Shawar et al., 1994).

In general, nonclassical class I MHC molecules have a more restricted tissue expression pattern and lower cell surface expression. Furthermore, their functions are generally not as clearly defined as their classical counterparts. Nevertheless, there is evidence suggesting that some have specialized antigen presenting functions (Shawar et al., 1994; Beckman et al., 1995). Due to the non-polymorphic nature of nonclassical class I MHC molecules, it is not surprising that the range of antigens that they present is rather limited. For example, murine H-2M3^a (formerly known as *Hmt*) has been demonstrated to bind and present N-formylated peptides derived from *Listeria monocytogenes* to CD8$^+$ T cells (Kurlander et al., 1992). Since prokaryotic peptides are formylated at the N-terminal, these nonclassical class I MHC are thought to play a role in controlling bacterial infection. Other nonclassical class I MHC molecules such as Qa-1 have also been demonstrated to present antigen to γδ T cells, but their exact functions remain elusive (Vidovic et al., 1989). For human nonclassical class I MHC molecules, only limited data are available regarding their functions. For HLA-G, due to its unique high level expression on trophoblasts, it has been postulated to have an important functional role in the maternal-fetal immune interaction (Carosella et al., 1996).

MHC-Like Molecules Encoded Outside the MHC Region

Although the tertiary structure of class I MHC molecules has been conserved in nature for the purpose of antigen presentation, evolution has also adopted and utilized this structure in some other ways. For example, the low affinity IgG neonatal Fc receptor (FcRn) and the Zinc binding protein (α-ZAG) have high degree of amino acid sequence identity to class I MHC molecules (Ueyama et al., 1991; Ahouse et al., 1993). As well as being highly homologous at the amino acid level, X-ray crystallography analysis has confirmed their class I MHC-like folding, with the organization of the extracellular domains into α1, α2 and α3 domains (Burmeister et al., 1994; Sánchez et al., 1999). Interestingly, the genes encoding these proteins are not linked to MHC. In fact, their genetic loci are actually located in different chromosomes. However, even with their class I MHC-like nature, none of these proteins have been implicated in presenting antigens to T cells. This is expected since the groove corresponding to the peptide binding groove of class I MHC molecules in FcRn, is closed, and α-ZAG is not associated with β_2m (Burmeister et al., 1994; Sánchez et al., 1999).

Not all of the non-MHC encoded class I MHC-like molecules are incapable of presenting antigens to T cells. For example, a group of non-MHC encoded antigen presenting molecules belonging to the CD1 family have been described in both human and mice (Blumberg et al., 1995). To date, only five human CD1 (CD1a, b, c, d and e) and two murine CD1 (mCD1.1 and mCD1.2) molecules have been detected (Blumberg et al., 1995). The human and murine CD1 genes are encoded on chromosome 3 and chromosome 1, respectively (Blumberg et al., 1995). In addition, the murine CD1.1 crystallographic data shows that it adopts a tertiary structure similar to that of classical class I MHC molecules and associates with β_2m (Zeng et al., 1997). Since their discovery in 1979, the functional relevance of CD1 molecules has only recently become clear. It seems that the immune system utilizes these molecules to present hydrophobic microbial glycolipid antigens to a sub-class of T cells called natural T cells (NKT) (Beckman et al., 1994). Since NKT cells can secrete large amounts of IL-4 and/or IFN-γ over a short period of time, it is speculated that CD1 may play a role in determining the direction and type of T cell responses, e.g.Th1 and Th2 responses (Yoshimoto et al., 1995).

C. Natural Killer Cells: Historical Perspective

According to the "genetic laws of classical transplantation", in inbred animals, parental grafts are accepted by F_1 offspring. This is due to each parent contributes half of the MHC locus and that MHC expression is co-dominant in the F_1 offspring. Therefore, the parental grafts are not considered foreign by the F_1 immune system. During the mid-1960's, Cudkowicz and colleagues demonstrated that B6 (H-2^b) derived bone marrow cells (BMCs) failed to grow in lethally irradiated B6xC3H (H-$2^{b/k}$) F_1 offspring (Cudkowicz et al., 1964); a phenomenon now commonly referred to as hybrid resistance. In the early

1970's, it was demonstrated that this phenomenon could be eliminated by treating the F_1 mice with ^{89}Sr, an element which selectively destroys the bone marrow microenvironment (Bennett. 1973). This observation therefore led to the concept that hybrid resistance was mediated by "marrow-dependent" effector cells, or "M-cells". However, the true nature of these M-cells was unknown at the time. Therefore, the hybrid resistance phenomenon mediated by these M-cells created a serious conflict with the well accepted self and non-self concept of classical transplantation, commonly observed with skin grafts.

During the mid-1970s, immunologists had noticed that in the absence of immunization, a unique population of mouse lymphoid cells exhibit high cytotoxic activity against a variety of allogeneic and syngeneic tumor cell lines (Herberman et al., 1975; Kiessling et al., 1975). This subset of lymphocytes seemed to lyse target cells in a MHC-independent manner. With such a high natural cytotoxic activity, these cells were designated as "N-cells". Furthermore, this lymphoid population could be detected in athymic mice (Herberman et al., 1975), suggesting they are indeed different from T cells which require a thymus for full maturation. Therefore, these N-cells were different from cytotoxic T lymphocytes, which only lyse target in the context of class I MHC loaded with foreign antigens. As in hybrid resistance, the existent of such a lymphoid population was also difficult to reconcile with the self and non-self concept. For instance, with the MHC-independent killing, how do these cells discriminate self from non-self?

With the development of different mutant mouse strains and antibody reagents which specifically recognize distinct cell populations, it is now clear that hybrid resistance is mediated by the lymphoid cells described by Herberman and Kiessling. Thus, M-cell and N-cell are in fact the same, and are now known as NK cells. The lack of MHC-restriction of NK cells can be explained by the fact that they do not rearrange their TCR genes. As a result, they undergo normal development in the absence of functional recombinant activator genes, Rag-1 and Rag-2, and in SCID mice (Dorshkind et al., 1985; Mombaerts et al., 1992; Shakai et al., 1992). Furthermore, NK cells tend to possess spontaneous cytotoxic activity against transformed and virally infected cells (Trinchieri. 1989). To facilitate the study of NK cells, at the Fifth International NK workshop, they have been finally defined as large granular lymphocytes (LGL) which exhibit an Ig^-, $CD3^-$, TCR^- and $CD16^+$ ($FcR_\gamma III$) phenotype. In addition, CD56 and NK1.1 antigens are also expressed by human and murine NK cells (C57BL/6 strain), respectively (Hercend and Schmidt. 1988). Functionally, NK cells are known to mediate cytotoxicity in the absence of class I or II MHC molecules and without prior sensitization. However, with the lack of Ig, TCR expression by NK cells, and their ability to mediate MHC-nonrestricted killing just adds to the confusion of how NK cells determine what is self and non-self.

D. Missing Self Hypothesis

Since NK cells do not express either Ig receptors or TCR, it was rather intriguing as to how they discriminate self from non-self. Why do NK cells not spontaneously lyse normal healthy cells if they do not use TCR as a means to discriminate self from non-self? What are the underlying mechanisms which suppress the cytotoxicity of natural killer cells? In the mid-1980s, Kärre and colleagues observed an inverse correlation of class I MHC expression by tumor cell lines and their sensitivity to NK cell cytotoxicity (Kärre et al., 1986). This observation strongly suggested that class I MHC molecules can modulate NK cell cytotoxic activity. Data obtained using tumor cells in *in vivo* systems also suggested the same inverse relationship. For example, a β_2m deficient (and thus class I MHC deficient) EL-4 T lymphoma mutant was unable to grow and metastasize in syngeneic B6 *nu/nu* mice which lack T cells, but retain functional NK cells. However, upon re-introduction of β_2m expression and thus restoration of class I MHC expression, *nu/nu* mice failed to reject the tumor (Kärre et al., 1986; Glas et al., 1992). Therefore, both *in vitro* and *in vivo* experiments supported the notion that although NK cells express no TCRs, their cytotoxic activities are clearly influenced by class I MHC molecules. A similar inverse relationship between NK cell killing and class I MHC expression is observed in human NK cells. By transfecting CIR or 721.221 cell lines with HLA-A, B and C, it was demonstrated that expression of these class I MHC molecules was sufficient to change the parental cell phenotype from NK cell sensitive to NK cell resistant (Shimizu and Demars. 1989; Storkus et al., 1989). The importance of class I MHC molecules in this inhibition is further strengthened by the fact that HLA-A2 with a single mutation in its $\alpha 1$ domain failed to induce the inhibitory effect (Storkus et al., 1989). This demonstrated the direct involvement of the class I MHC extracellular region in modulating NK cell cytotoxic activity. Therefore, these results suggested that whatever the mechanism of inhibition is, NK cells must recognize the class I MHC molecule in a specific manner.

In an attempt to explain the inverse relationship of target cell class I MHC expression and its NK cell sensitivity, Kärre and colleagues have put forward the "missing self" hypothesis (Ljunggren and Kärre. 1990). The core of this hypothesis is that NK cells lyse target cells with reduced expression of self-class I MHC molecules. In essence, Ljunggren and Kärre suggest that NK cells determine self and non-self by monitoring self class I MHC levels. Consequently, with the universal presence of self-class I MHC molecules, NK cell activity is inhibited and unable to lyse normal healthy cells. Only under circumstances of aberrant reduced self-class I MHC expression, caused by viral infection or cell transformation, can target cells be lysed by NK cells. Therefore, this hypothesis also explains why NK cells have such a high cytotoxic activity against virally infected and transformed cells. At the time, Ljunggren and Kärre further proposed two basic preliminary models, the "target interference" model and the "effector inhibition" model to explain the direct inhibitory effect of class I MHC molecules. The target interference model proposes that NK cells are activated upon interacting with some "triggering structures" on target cells. However, high class I MHC expression simply masks the triggering structure on the target cells and thus prevents the triggering structure from engaging the "triggering receptor" on NK cells. As a result, no activation of NK cells can take place (Fig. 1-1A). In contrast, the effector inhibition model suggests that triggering or activation of NK cells occurs by default. However, such triggering events are prevented by engagement of class I MHC-specific inhibitory receptors. Upon interacting with self-class I MHC molecules, these putative inhibitory receptors transduce dominant negative signals and shut down NK cell functions. This model, therefore, requires that NK cells express class I MHC-specific inhibitory receptors (Fig. 1-1B). With the recent discovery of various NK cell receptors in both humans and mice, the effector inhibition model is now widely viewed as the more plausible model for the missing self hypothesis.

According to Kärre, the phenomenon of hybrid resistance is one of the main driving forces for the development of this hypothesis (Kärre. 1997). So not surprisingly, the missing self hypothesis seems to be able to explain the fundamental properties of NK cells and to provide an underlying mechanism for the NK cell-mediated hybrid resistance phenomenon. For example, it has been demonstrated experimentally that if BMC grafts do not express all of the host class I MHC molecules, inhibitory signals will not be delivered to all subsets of NK cells (Yu et al., 1996). As a result, rejection of parental BMCs by F_1 offspring will take place typically mediated by a subset of host NK cells. An important aspect of the missing self hypothesis as an explanation for hybrid resistance is that not all NK cells are proposed to, and indeed do not, express the same set of self class I MHC-specific inhibitory receptors.

E. Natural Killer Cell Regulatory Receptors

Direct evidence for the existence of NK cell inhibitory receptors, predicted by the missing self hypothesis, was obtained almost a decade after its original proposal. By isolating NK cell subsets expressing the Ly-49A receptor, the only known NK cell inhibitory receptor at the time, Karlhofer and colleagues were able to show a correlation between D^d and an $H\text{-}2^k$ product expression by target cells with resistance to Ly-49A$^+$ NK cell lysis (Karlhofer et al., 1992). The direct involvement of class I MHC molecules in protecting target cells from Ly-49A$^+$ NK cell lysis was further demonstrated by the observation that anti-Ly-49A and anti-D^d mAb against the D^d $\alpha1/\alpha2$ domains can restore the cytotoxic activity of Ly-49A$^+$ NK cells (Karlhofer et al., 1992). This strongly suggests the inhibitory effect is a result of direct interaction between Ly-49A and the $\alpha1/\alpha2$ domains of D^d. However, the first evidence demonstrating the direct physical interaction of Ly-49A and D^d was provided by Kane (Kane. 1994). Using an *in vitro* cell adhesion system, it was further shown that in addition to D^d, the uncharacterized H-2^k product that is suggested to inhibit Ly-49A$^+$ NK cell function in the Karlhofer study, is D^k (Kane. 1994). Since then, the list of receptors possessing similar NK cell regulatory functions has been growing exponentially.

On the basis of their amino acid sequence identity, NK cell inhibitory receptors are classified into two superfamilies; the animal C-type lectin and the immunoglobulin (Ig) superfamilies (Fig. 1-2). NK cell regulatory receptors belonging to both superfamilies have been described and characterized in both humans and mice (Lanier and Phillips. 1996; Lanier. 1997a; Lanier. 1997b; Lanier. 1998b). For example, the murine NK cell regulatory receptors such as CD94/NKG2, Ly-49 and NKRP-1 belong to the animal C-type lectin superfamily. In contrast, 2B4 and gp49 are classified under the Ig superfamily. However, not all of these NK cell regulatory receptors recognize class I MHC molecules as their ligands. In the murine system, only receptors belonging to the animal C-type lectin superfamily are known to interact with class I MHC molecules. These include the Ly-49 and CD94/NKG2 receptor families. In contrast, class I MHC-specific regulatory receptors from both superfamilies have been described in humans (Lanier. 1998b). For example, human CD94/NKG2 receptors have been identified and demonstrated to recognize nonclassical class I HLA-E bound with signal sequence-derived peptides of classical class I MHC proteins (Braud et al., 1998; Borrego et al., 1998). Although a truncated form of human Ly-49-like molecule has been cloned (Westgaard et al., 1998), no functional human Ly-49 receptor counterpart has been identified.

The first HLA-specific NK cell receptors identified in humans were those belonging to the Ig superfamily. These NK cell receptors exhibit the classical Ig-fold structure and are generally expressed on the cell surface as monomers, in some cases as disulfide-linked dimers (Lanier and Phillips. 1996; Lanier. 1998b). These receptors are referred to as the killer cell inhibitory receptors (KIRs). On the basis of the number of Ig domains in their extracellular region, these receptors are further classified into two sub-families; KIR2D and KIR3D. The former has two Ig domains in their extracellular region, whereas the latter has three.

It is now clear that among all of the class I MHC-specific receptors, not all of them function as NK cell inhibitory receptors, as within each NK cell regulatory receptor family, there are members that seem to activate NK cell functions upon engaging class I MHC molecules (Lanier. 1998b). Therefore, the regulation of NK cell activities is much more complicated than previously thought. For example, the ability of NK cell activating receptors to engage class I MHC molecules is inconsistent with the missing self hypothesis.

NK Cell Receptors of the Animal C-type Lectin Superfamily and the NK Gene Complex (NKC)

The animal C-type lectins are proteins that bind carbohydrate moieties in a Ca^{2+}-dependent manner, mediated by the conserved carbohydrate recognition domain (CRD). Based on amino acid sequence identities, all NK cell regulatory receptors of this C-type lectin superfamily are predicted to possess a membrane distal domain resembling a CRD (Yokoyama et al., 1989). However, a distinctive feature of these animal C-type lectin NK cell receptors is that they are all type II transmembrane glycoproteins, and expressed on the cell surface either as disulfide-linked homodimers or heterodimers. Based on amino acid sequence identities, receptors of this NK cell receptor family are further divided into multiple sub-families, such as the NKR-P1, NKG2 and Ly-49 gene families (Fig. 1-2).

However, only those of the NKG2 and Ly-49 gene families have been clearly demonstrated to recognize class I MHC molecules as their ligands (Takei et al., 1997; Braud et al., 1998; Borrego et al., 1998; Vance et al., 1998). In the murine system, NKR-P1 consists of three family members, NKR-P1A, B and C, in which NKRP-1C encodes for the NK1.1 marker (Ryan and Seaman. 1997), whereas in humans, only NKR-P1A has been described (Lanier et al., 1994). For the NKG2 family, including the splice variants, at least five family members, NKG2A, C, E, and D/F have been identified in humans (Lanier. 1998b). In mice, at least three NKG2 genes have been cloned (Vance et al., 1998; Lohwasser et al., 1999; Silver et al., 1999). NKG2 subunits are expressed on the cell surface as disulfide-linked heterodimers with another animal C-type lectin receptor subunit, CD94 (Carretero et al., 1997; Lanier. 1998b). Interestingly, CD94 itself can be expressed independently as a homodimer in some transfectants (Carretero et al., 1997; Vance et al., 1998), however, the functional relevance of this is not known.

The genes encoding animal C-type lectin NK cell receptors are clustered in an approximately two megabase segment of murine chromosome 6 which is referred to as the NK gene complex (NKC) (Brown et al., 1997a; Brown et al., 1997b). In addition to the *Ly55 (Nkrp1)*, *Ly49* and *Nkg2* multigene families, other individual C-type lectin NK cell receptor genes such as *Cd69,* and *Cd94* also map to the NKC (Brown et al., 1997; Ho et al., 1998; Vance et al., 1997). Related genes are also located on human chromosome 12p13 (Suto et al., 1997), suggesting the common evolutionary history of these animal C-type lectin NK cell regulatory genes at these loci. Several genetic loci linked to viral resistance phenotypes have been mapped to the NKC, for example, the *Cmv1* and *Rmp1* loci are known to influence the pathogenesis caused by mouse cytomegalovirus (CMV) and ectromelia virus (mousepox), respectively (Delano and Brownstein. 1995; Forbes et al., 1997). Recent data suggest that the *Cmv1* locus is in fact closely linked to loci encoding the Ly-49 multigene family (Forbes et al., 1997). This is not surprising since the CMV genome encodes a class I MHC homolog and a protein that down regulates host class I

MHC expression (Thale et al., 1995; Rowlinson et al., 1996); it is only logical that host resistance to this virus is dependent on NK cells. Therefore, these results indicate the important role that NK cells play in controlling viral and parasite infections.

F. The Murine Ly-49 NK Cell Receptor Family

The first NK cell inhibitory receptor to be described and characterized was the murine Ly-49A receptor, identified on the T lymphoma cell line, EL-4 (Nagasawa et al., 1987). Since then, excluding the splice variants, nine family members of Ly-49, Ly-49A - I in total, have been reported (Takei et al., 1997). However, there is strong evidence indicating that there are other Ly-49 molecules which remain to be described and characterized (McQueen et al., 1998; Kane, unpublished data). Most Ly-49 receptors bear a high degree of identity to each other at both the amino acid and nucleotide levels, except Ly-49B, which has a low degree of amino acid sequence identity to the other Ly-49 family members.

The structure of Ly-49 receptors can be roughly divided into 4 domains. The carbohydrate recognition domain (CRD) and the stalk region constitute the extracellular portion of the receptor. This extracellular region is then followed by the transmembrane region and the cytoplasmic tail. As in other animal C-type lectins, all Ly-49 receptors possess four conserved Cys residues in its CRD (Yokoyama et al., 1989) and are expressed as disulfide-linked homodimers on the cell surface (Nagasawa et al., 1987). Based on the limited available data, no heterodimerization between Ly-49 family members has been detected (Brennan et al., 1996b). However, whether closely related family members such as Ly-49C and I, which show 98% identity to each other (Sundbäck et al., 1996), can heterodimerize remains unknown.

Due to the lack of Ly-49-specific reagents, the distribution and expression of most Ly-49 receptors on NK cells have not been determined. However, using available antibody reagents, it appears that each Ly-49 receptor is expressed on a distinct but overlapping subset of NK cells (Mason et al., 1995; Mason et al., 1996). For instance, Ly-49A and G2 are detected on approximately 20% and 50% of the total NK cells, respectively (Mason et al., 1995; Yokoyama. 1995). A similar distribution is also seen with Ly-49C expression (Yu et al., 1996). As a result of this non-uniform distribution of Ly-49 receptors, the NK cell subsets are extremely heterogeneous. For example, within the Ly-49A$^+$ NK cell population, they can be further fractionated into Ly-49A$^+$G$^+$ and Ly-49A$^+$G$^-$. This heterogeneity is further compounded by the non-uniform expression of receptors belonging to other NK cell receptor families. Essentially, it is difficult, if not impossible to isolate an NK cell population that expresses a single Ly-49 family member.

Interaction of Ly-49 Receptors and Class I MHC Molecules

Ly-49A was the first NK cell inhibitory receptor demonstrated to physically interact with class I MHC H-2D^d and D^k (Kane. 1994; Daniels et al., 1994a). Isolated Ly-49A$^+$ NK cells were demonstrated to be unable to lyse H-2D^d and H-2^k target cells (Karlhofer et al., 1992). The *in vitro* physical interaction experiments therefore correlated with those obtained with *in vitro* killing assays, suggesting that engagement of Ly-49A with H-2D^d initiates and delivers inhibitory signals to NK cells preventing them from lysing D^{d+} target cells. More recently, depending on the genotype of the target cells, other Ly-49 family members such as Ly-49C and Ly-49G2 have been demonstrated to inhibit NK cell functions (Mason et al., 1995; Yu et al., 1996). For example, similar to Ly-49A, Ly-49G2 also recognizes H-2D^d in addition to D^r and possibly L^d (Mason et al., 1995; Thaddeus, et al., 1999). Therefore, it seems that not only the distribution, but also the class I MHC ligand specificities of Ly-49 members can partially overlap. Since mAbs are not available for Ly-49B, E and F, their expression, distribution and class I MHC ligand specificities have not yet been determined.

It is now believed that Ly-49 receptors recognize the $\alpha 1$ and $\alpha 2$ domains of their cognate class I MHC ligands. For example, anti-D^d ($\alpha 1/\alpha 2$ domains) but not anti-D^d ($\alpha 3$

domain)-specific mAbs reversed the inhibitory effect mediated through Ly-49A (Karlhofer et al., 1992; Matsumoto et al., 1998). Furthermore, domain swapping experiments demonstrated that the $\alpha2$ domain of D^d restricted the allelic specificity of Ly-49A (Sundbäck et al., 1998). Specific recognition of the $\alpha1$ and $\alpha2$ domains of class I MHC has also been demonstrated for Ly-49C and G2 (Brennan et al., 1994; Mason et al., 1995).

With respect to the recognition of class I MHC $\alpha1$ and $\alpha2$ domains by Ly-49 receptors, the important question was whether this interaction was also dependent on the bound peptides presented by the class I MHC molecules. Peptide-dependency could be a potential mechanism for NK cells to discriminate self from non-self. However, the first evidence to suggest otherwise was provided by Correa and colleagues (Correa and Raulet. 1995). It seems that the interaction and initiation of inhibitory signals for Ly-49A are peptide-dependent, but not peptide specific (Correa and Raulet. 1995; Orihuela et al., 1996). Therefore, as long as the class I MHC is stabilized on the cell surface by β_2m and peptides, Ly-49A does not seem to recognize the contents of the peptide binding groove. Nevertheless, to generalize this observation to other Ly-49 family members must be done with caution, as it was recently reported that Ly-49C is capable of recognizing empty K^b (Su et al., 1998).

Since Ly-49 receptors exhibit amino acid sequence identity to animal C-type lectins, it was suggested that the *N*-linked carbohydrates on class I MHC molecules could be involved in this interaction. The fact that the Ly-49 receptor is a homodimer, and interacts with the $\alpha1$ and $\alpha2$ domains of class I MHC, and the fact that there are two conserved *N*-linked glycosylation sites on these domains, suggests the intriguing possibility that Ly-49 receptors might recognize *N*-linked carbohydrate moieties on class I MHC molecules. It is important to note that amino acid sequence analysis reveals that the CRDs of Ly-49 differ from the CRDs of other animal C-type lectins, as a subset of the highly conserved residues found in the other CRDs are not found in Ly-49 CRDs. Nevertheless, early evidence provided by Yokoyama and colleagues demonstrated that the Ly-49A CRD is indeed functional and can bind a sulfated-fucose containing polysaccharide (Daniels et al., 1994b). This carbohydrate-dependent interaction is also seen with Ly-49C interaction with H-2^{s} product(s) (Brennan et al., 1995). Therefore, it was generally accepted that the *N*-linked glycosylation of class I MHC is needed for this type of receptor-ligand interaction. Furthermore, domain swapping experiments indicated that the Ly-49 CRD is needed for its ligand binding. However, the specificity of class I MHC interaction with Ly-49C also requires the participation of 19 amino acids adjacent to the CRD, as H-$2^{b,s}$-expressing cells failed to bind to Ly-49C without this segment of amino acids (Brennan et al., 1996b). Although there is ample evidence suggesting that the CRD can interact with carbohydrate moieties, whether class I MHC *N*-linked carbohydrates can directly influence the interaction with the Ly-49 receptor remains unclear. In this regard, recent published data offered polarized view points regarding the importance of class I MHC *N*-linked carbohydrate moieties in its interaction with Ly-49 receptors. By mutating the two conserved *N*-linked glycosylation sites, Yokoyama and colleagues showed that D^{d} *N*-linked carbohydrates are not required in Ly-49A interaction (Matsumoto et al., 1998). In contrast, Takei and colleagues demonstrated that the N176 *N*-linked glycosylation site is critical in this receptor-ligand interaction (Lian et al., 1998). As a result, the exact role of class I MHC *N*-linked carbohydrates in this receptor-ligand interaction remains controversial.

Diversity of Ly-49 Receptors

The complexity of the Ly-49 NK cell receptor family not only arises from the fact that there are multiple members within the family. In addition, polymorphism is also seen in certain family members, for example, the Ly-49A alleles from B6 (Ly-$49A^{B6}$) and Balb/c (Ly-$49A^{Balb/c}$) exhibit 98% identity (Held et al., 1995). Nevertheless, this small difference is significant enough to allow a specific mAb, A1, to be raised against Ly-49^{B6} in Balb/c mice (Nagasawa et al., 1987). The functional significance of this Ly-49 allelic diversity is currently unknown. However, since many polymorphic residues defining Ly-49 alleles reside in the extracellular portion of Ly-49 molecules, it is possible such differences could influence Ly-49 receptor affinities to certain class I MHC allelic products. For instance, Ly-49C is closely related to Ly-49I (Sundbäck et al., 1996), however, cell-cell adhesion assays showed that Ly-49C, but not Ly-49I, can interact with a wide range of H-2 products (Brennanet al., 1996a). Therefore, it is possible that small allelic differences can influence the specificity of Ly-49 receptors. Based on single cell polymerase chain reaction, Held and Kunz have shown that most NK cells from F_1 hybrid mice only express one of the alleles of a particular Ly-49 receptor (Held and Kunz. 1998), as NK cells isolated from B6xBalb/c F_1 express either the B6 or Balb/c allele of Ly-49A. So far the functional relevance of this phenomenon has not been addressed. It is possible that NK cell subsets which express different alleles of the same Ly-49 gene might function differently.

Ly-49 Receptor Signaling Pathways

According to the missing self hypothesis, NK cell triggering events have to be intercepted by dominant negative signals mediated by inhibitory receptors. The first glimpse of the molecular basis of the NK cell inhibitory effect came from the study of human KIR2DL p58 (Burshtyn et al., 1996). Co-immunoprecipitation experiments showed that cross linking of human KIR by mAb induced the association of the hematopoietic cell phosphatase, SHP-1, with the inhibitory receptor (Burshtyn et al., 1996). It appears that SHP-1 interrupts the early signaling events which are required for NK cell activation by dephosphorylating the membrane proximal tyrosine, phosphorylated by membrane tyrosine kinases. It was further demonstrated that the association of SHP-1 with NK cell inhibitory receptors requires the phosphorylation of the Tyr residue of the inhibitory receptor cytoplasmic tail, and this Tyr residue lies within consensus sequences (V/LXYXXL) called the immunoreceptor tyrosine-base inhibitory motif (ITIM). This motif is involved in signaling for various cell surface inhibitory molecules (Vély and Viver. 1997). The molecular basis of this inhibitory mechanism seems to be conserved in NK cells, as inhibitory receptors of different NK cell receptor families possess either one or two ITIM in their cytoplasmic tails (Lanier. 1998b). In the case of Ly-49 receptors, association of SHP-1 with Ly-49A and Ly-49G2 has also been demonstrated (Mason et al., 1997; Nakamura et al., 1997). However, it is important to note that there might be some redundancy within the system. For example, Ly-49A$^+$ NK cells isolated from SHP-1 deficient, motheaten *Me/Me* mice, still can mediate inhibition through class I MHC molecules (Nakamura et al., 1997), suggesting that SHP-1 is not absolutely required for the disruption of the early signaling events in NK cells. However, the ITIM motif in the cytoplasmic tail is essential for inhibitory receptor functions. Mutagenesis studies demonstrated that changing the Tyr residue to Phe within the ITIM essentially eliminates the biological function of inhibitory receptors (Mason et al., 1997).

By examining the cytoplasmic tail amino acid sequences of Ly-49 receptors, it was realized that Ly-49D and H do not possess any ITIMs (Ryan and Seaman. 1997). Interestingly, both of these receptors have a positively charged residue in their transmembrane regions. At first, the function of these ITIM-less Ly-49 receptors was not known. Furthermore, it was quite puzzling as to how they transduce signals without any ITIMs and how they were able to remain stable in the membrane with the positively charge residue in the hydrophobic transmembrane regions. It is now clear that these receptors function to activate, not to inhibit, NK cell functions. Due to the lack of anti-Ly-49H reagents, only Ly-49D has been clearly shown to activate NK cell cytotoxic activity (Mason et al., 1996; Thaddeus et al., 1999). Nevertheless, it is expected that Ly-49H also possesses a similar biological function. As for class I MHC specificity, Ly-49D has been shown to recognize D^d, as H-2D^d transfectants are capable of activating the Ly-49D$^+$ NK cells (Nakamura et al., 1999; Thaddeus et al., 1999). Therefore, the class I MHC specificity of this activating NK cell receptor is also overlapping with those of inhibitory receptors such as Ly-49A and G2.

The inability of activating Ly-49 receptors to signal through their cytoplasmic tails is overcome by their association with a signaling adaptor molecule called DAP-12 (Colonna. 1998a; McVicar et al., 1998; Smith et al., 1998). DAP-12 is a 12 kDa cell surface disulfide-linked homodimer that possesses a negatively charged residue in its transmembrane region. As a result, this enables DAP-12 to physically associate with activating receptors (Lanier et al., 1998c). More importantly, DAP-12 itself possesses an immunoreceptor tyrosine-base activating motif (ITAM) in its short cytoplasmic tail (Lanier et al., 1998c). Upon engaging the activating Ly-49 receptors, activating signal is initiated from this associated component (Lanier et al., 1998c; McVicar et al., 1998), presumably through recruitment of tyrosine kinases.

As mentioned previously, within each NK cell regulatory receptor family, there are both class I MHC-specific inhibitory and activating receptors. Evidence suggests that evolution conserves the molecular basis for generation of these inhibitory and activating signals, as the inhibitory receptors of CD94/NKG2, Ly-49 and KIRs all possess the ITIM and are associated with SHP-1 upon engagement. Whereas, their activating receptor counterparts all lack the ITIM in their cytoplasmic tails, and possess one or two positively charged residue within the transmembrane region which allows them to associate with DAP-12.

The existence and the need for such class I MHC-specific activating NK cell receptors is an unresolved issue. In fact, the missing self hypothesis is inadequate to explain the need for these class I MHC-specific activating receptors. Nevertheless, current opinion is that NK cell cytotoxic activity is determined by the signals generated by both activating and inhibitory receptors.

G. Down Regulation of NK Cell Receptors and Receptor Calibration Model

In order to prevent self-reactivity, each NK cell must express at least one inhibitory receptor which recognizes self class I MHC molecules. However, high level of self-inhibitory receptors expression is obviously detrimental and will render all NK cells functionally useless. For instance, the Ly-49A$^+$ NK cell subsets in H-2^d (D^{d+}) mice will constantly receive the dominant inhibitory signals and override any potential triggering events. The problem is further compounded by the expression of other H-2^d-specific Ly-49 inhibitory receptors such as Ly-49C and G2. Therefore, by virtue of their overlapping expression patterns, most, if not all of the NK cell subsets will not be activated if these self-inhibitory receptors are expressed at high levels. At the other end of the spectrum, expression of low affinity self-class I MHC-specific inhibitory receptors is also undesirable, since NK cells will be spontaneously lytic against all cells. In this instance, expression of higher levels of the self-class I MHC recognizing inhibitory receptors has to be induced. To achieve this delicate balance, it is only logical to hypothesize that NK cells have to go through some form of "education" or "selection" process. Because most NK cells express multiple inhibitory receptors specific for self-class I MHC, NK cell clonal deletion, if it occurs, would result in removal of most NK cells. In this respect, the NK cell selection process is thought to be different from that of T cells, which typically undergo clonal deletion to remove self-reactive T cells.

To resolve this issue, Kärre and colleagues have proposed that a cellular adaptation process is in place for NK cells to "educate" themselves. They suggested that NK cells calibrate their inhibitory receptor expression levels to somatically adapt to the allele and the level of class I MHC products expressed by their host (Olsson et al., 1995). In other words, the expression of the self-reactive Ly-49 inhibitory receptor is influenced and regulated by host class I MHC molecules. This is supported by the observation that Ly-49A expression is down regulated in Balb/c (H-2^d) mice expressing the ligand D^d, but the percentage of Ly-49A$^+$ NK cells remains the same (Karlhofer et al., 1994; Olsson et al., 1995; Johansson et al., 1998). However, the strongest evidence which directly demonstrated that class I MHC molecules influence Ly-49 receptor expression was provided by the studies of transgenic mice. Introduction of H-2D^d into H-2^b mice (also known as D8 mice) alone was sufficient to alter the Ly-49A expression pattern (Olsson et al., 1995). This Ly-49A down regulation process only requires the presence of the $\alpha 1$ and $\alpha 2$ but not the $\alpha 3$ domains of H-2D^d (Johansson et al., 1998). Therefore, these data suggest that interaction of Ly-49 with its class I MHC ligands is a critical step in the receptor modulation process.

With the reduction in Ly-49A expression, it is not surprising that the functional phenotype of these D8 derived NK cells is also altered (Olsson et al., 1995). For example, these NK cells failed to reject D^d-expressing tumors and BMCs. In addition, as compared to the parental B6 mice, tolerance toward autologous B6 BMCs is not observed in these D8 mice. Therefore, introduction of a single allele of class I MHC is sufficient to alter the function of NK cells. However, the interesting observation is that Ly-49A$^+$ NK cells derived from D8 mice seem to have a greater capacity to monitor minor changes in H-2D^d expression levels. They are capable of lysing low but not high H-2D^d expressing cell lines (Olsson et al., 1995; Olsson et al., 1997). Thus, functionally it will be more difficult to inhibit Ly-49A^{low} NK cell cytotoxic activity with suboptimal levels of H-2D^d.

According to the receptor calibration model, the total avidity of the inhibitory receptor interaction is the deciding factor that determines whether the triggering signals can be overridden or not. Therefore, depending on the strength of the triggering signals, there is an avidity threshold that the inhibitory interaction must reach in order to inhibit lysis of target cells. Multiple factors are contributing to the avidity of this interaction, including the total number of inhibitory receptors engaged at a give time point, which itself is influenced by the quantity of class I MHC molecules available for interacting with the inhibitory receptors. In addition, the affinity of the class I MHC and Ly-49 interaction itself certainly contributes to the avidity of the interaction.

It is not difficult to imagine that modulation of self-reactive inhibitory receptor density on NK cells will lead to heightened sensitivity to changes in class I MHC levels. As a result, these NK cells are better suited to detect minor aberrant class I MHC expression and to function more efficiently in immune surveillance.

H. Regulation of NK Cell Functions by Nonclassical Class I MHC

With mounting evidence suggesting that classical class I MHC could regulate NK cell functions, it was generally suspected that nonclassical class I MHC might possess similar regulatory abilities. This is not an unreasonable assumption, given the fact that these nonclassical class I MHC molecules have been shown to have similar structural folding as classical class I MHC and to function as antigen presenting molecules. The first evidence that nonclassical class I MHC molecules may regulate NK cells came from the study of HLA-G and its inhibitory effect on human decidual NK cells (Chumbley et al., 1994). Since HLA-G is expressed on trophoblasts, it was suggested that HLA-G might function to suppress NK cell activity at the feto-maternal interface. As a result, it could play an important role in maternal tolerance of the fetus. However, the central role of HLA-G in inhibiting NK cell cytotoxic activity has recently come into question. For instance, it was found that co-transfection of HLA-E and the leader sequence of HLA-G in NK cell sensitive targets was sufficient to protect them from NK cell lysis (Braud et al., 1998; Borrego et al., 1998; Lanier. 1998b). It was further demonstrated that HLA-E loaded with HLA-G leader sequence could bind to the NK cell receptors belonging to the CD94/NKG2 family (Braud et al., 1998). Nevertheless, it is important to note that HLA-E itself is a nonclassical class I MHC molecule. Therefore, the data still support the notion that nonclassical class I MHC molecules can regulate NK cell functions. Although the direct involvement of HLA-G in regulating NK cell functions remains controversial, it has been reported that HLA-G is the ligand for receptors belonging to the Ig superfamily. For example, upon interacting with an Ig-like inhibitory receptor, ILT4, expressed on myeloid cells, an inhibitory signal is initiated (Colonna et al., 1998b). In addition, recent data has also indicated that HLA-G may also interact with another KIR called p49 expressed on decidual NK cells (Ponte et al., 1999). Therefore, the likelihood that HLA-G can directly regulate NK cell functions cannot be dismissed.

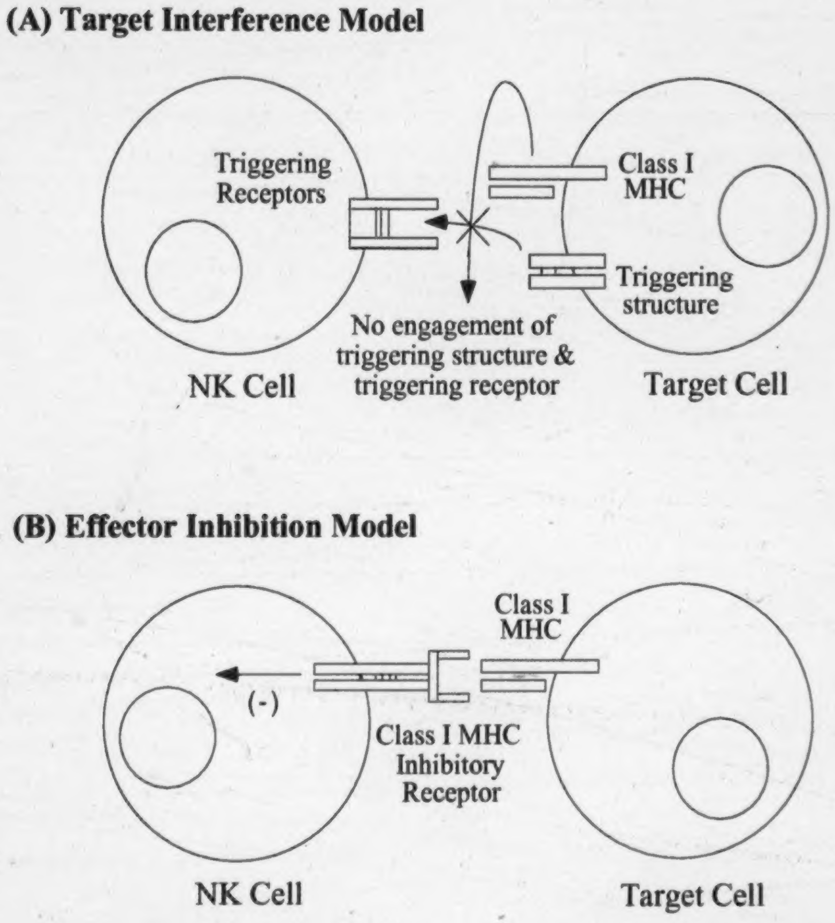
As for the murine system, a similar observation also has been made in some nonclassical class I MHC molecules. By using nonclassical class I MHC tetramers, the murine orthologue of HLA-E, Qa-1^b, has recently been shown to interact with CD94/NKG2A heterodimers on NK cells (Vance et al., 1998). This interaction also prevents the lysis of Qa-1^{b+} target cells loaded with leader sequences derived from other class I MHC molecules. These results are in agreement with those of the human studies, and firmly demonstrate the ability of nonclassical class I MHC to regulate NK cell functions. Therefore, these observations raise the possibility that other nonclassical class I MHC, including antigen presenting molecules encode outside the MHC region, also possess similar regulatory functions. In addition, these results seem to suggest that evolution has adopted different receptor families to interact with classical and nonclassical class I MHC molecules.

I. Rationale and Objectives

Since the cloning of the first murine Ly-49 NK cell regulatory receptor, intense studies have been focused on how these receptors interact with their class I MHC ligands. However, due to the overlapping expression patterns on various NK cell subsets (Mason et al., 1995; Mason et al., 1996), and the lack of anti-Ly-49-specific reagents, the studies of Ly-49 and class I MHC interaction remain difficult. Therefore, the first aim of this present study was to examine the Ly-49A and D^d interaction in an *in vitro* system that we have developed, where a number of parameters can be controlled. In order to examine Ly-49A and D^d interactions directly without the interference of other Ly-49 receptors, we isolated Ly-49A from EL-4 tumor cell line and examined its interaction with H-$2D^d$-expressing cells. Using this *in vitro* cell adhesion system, the influence of Ly-49A densities on its interaction with D^d-expressing cells was addressed. Others and we have evidence that Ly-49A can bind sulfated carbohydrates. Therefore, the possibility that H-$2D^d$ could be sulfated on its *N*-linked carbohydrates was also investigated. The effect of this post-translational modification on D^d interaction with Ly-49A was further examined also using the *in vitro* cell adhesion system.

Since the lack of anti-Ly49 mAb remains one of the major obstacles in studying Ly-49 receptor functions, the second aim of this present study was to generate anti-Ly-49-specific reagents. In this regard, two anti-Ly-49G2-specific mAbs secreting hybridomas have been successfully generated. With these novel mAbs, the expression of Ly-49G2 and the functions of Ly-$49G2^+$ NK cells were re-evaluated.

With the recent demonstration that nonclassical class I MHC molecules can regulate NK cell function, the final objective of this thesis project was to determine whether the third lineage of antigen presenting molecules, the CD1 family, might also possesses similar NK cell regulatory functions.



(A) Target Interference Model

Triggering Receptors
Class I MHC
Triggering structure
No engagement of triggering structure & triggering receptor
NK Cell
Target Cell

(B) Effector Inhibition Model

Class I MHC
(-)
Class I MHC Inhibitory Receptor
NK Cell
Target Cell

Figure 1-1. Schematic diagram of missing self hypothesis. Missing self hypothesis proposed that NK cells discriminate self and non-self by monitoring self-class I MHC expression on target cells. If the target cells express reduced level of self-class I MHC molecules, inhibition of NK cell cytotoxic activity is relieved and lysis of target cell proceeds. Two basic models; target interference model (A), and effector inhibition model (B) have been proposed by Kärre and colleagues to account for the missing self hypothesis.

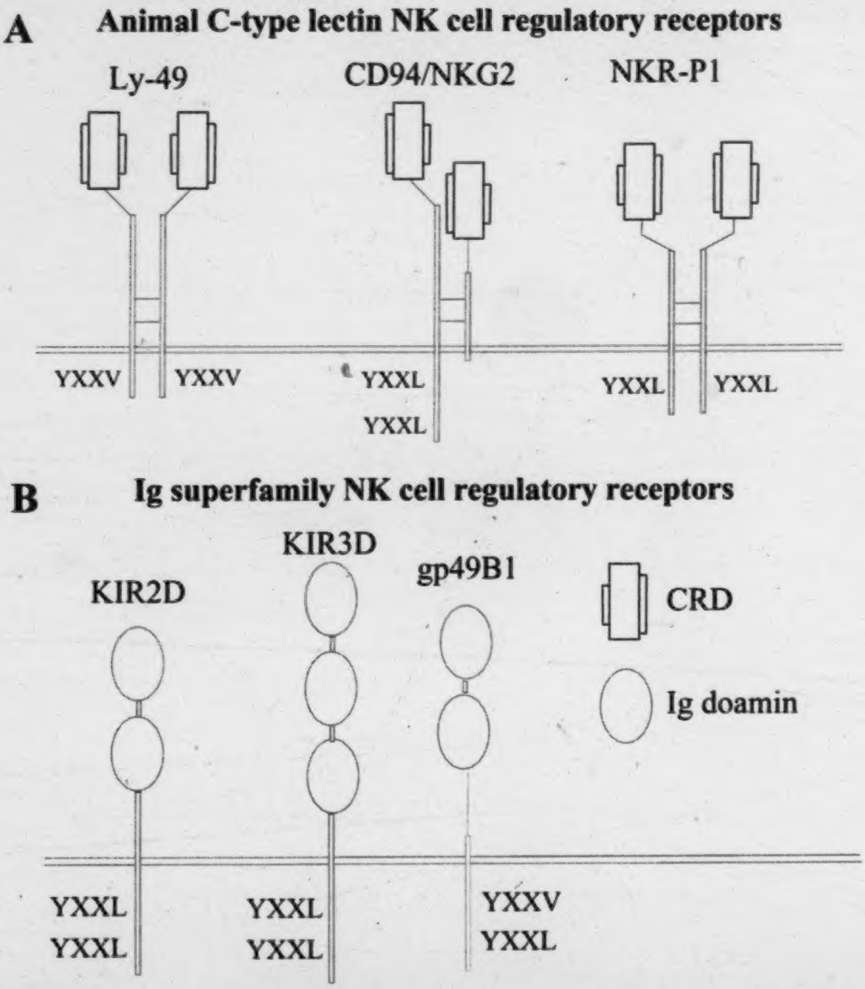

A
Animal C-type lectin NK cell regulatory receptors
Ly-49
CD94/NKG2
NKR-P1
YXXV
YXXV
YXXL
YXXL
YXXL
YXXL
B
Ig superfamily NK cell regulatory receptors
KIR2D
KIR3D
gp49B1
CRD
Ig doamin
YXXL
YXXL
YXXL
YXXL
YXXV
YXXL

Figure 1-2. Schematic diagram representation of NK cell regulatory receptors. Based on amino acid sequence identity, NK cell regulatory receptors are classified into two superfamilies; animal C-type lectin family (A) and Ig superfamily (B). The animal C-type lectin family consists of sub-families such as Ly-49, CD94/NKG2 and NKR-P1. Each of these receptors is expressed on the cell surface as type II transmembrane disulfide-linked homodimers (Ly-49 and NKR-P1) or heterodimers (CD94/NKG2), and each monomer subunit possesses a CRD. In contrast, NK cell regulatory receptors of the Ig superfamily, such as the human KIR2D and KIR3D, and the murine gp49B1 adopt the Ig-fold structure. The cytoplasmic tails of the inhibitory receptors in each family possess one or two ITIMs, where the inhibitory signal is believed to be initiated. In this diagram, only the inhibitory receptors in each subfamily are shown. However, activating receptors are also found in each sub-family, and they do not possess ITIM in their cytoplasmic tails. Furthermore, they also have one or two positively charged residues in their transmembrane region which allow them to associate with the signaling molecular adaptor, DAP-12 (see text).

CHAPTER II

MAJOR HISTOCOMPATIBILITY COMPLEX CLASS I-DEPENDENT CELL BINDING TO ISOLATED Ly-49A: EVIDENCE FOR HIGH-AVIDITY INTERACTION

(Most of the data presented in this chapter are published in the ***European Journal of Immunology (1996), 26:3219-3223.*** *Copyright (1996), The European Federation of Immunological Societies.)*

A. Introduction

The expression level of a particular murine animal C-type lectin-like Ly-49 NK cell receptor is strongly influenced by the host H-2 background (Karlhofer et al., 1994; Salcedo et al., 1997; Kåse et al., 1998). For instance, the level of Ly-49A expression is reduced in mouse strains which express one of its cognate class I MHC ligands, H-2D^d (Karlhofer et al., 1994; Salcedo et al., 1997). The role that class I MHC molecules plays in this receptor down regulation phenomenon is most convincingly demonstrated by H-2D^d transgenic mice studies. Introduction of a D^d transgene into B6 mice leads to a substantial reduction of the Ly-49A expression level (Johansson et al., 1998). Similar down regulation of Ly-49C and Ly-49G2 expression levels by their class I MHC ligands, K^b and D^d respectively, have also been described (Anderson et al., 1998; Johansson et al., 1998; Sundbäck et al., 1996). The need for such down regulation becomes obvious when considering the strength and amount of inhibitory signals that will be received by NK cells express high level of self-reactive inhibitory receptors. Thus, these self-inhibitory receptors are down regulated to a level in order to prevent these NK cells from becoming functionally useless. However, with the reduced self-inhibitory receptor expression level, these NK cells are more sensitive to slight reductions in host class I MHC level. This is the essence of the calibration model proposed by Kärre and colleagues as discussed in chapter I.

The strongest evidence which supports the receptor calibration model is again provided by the transgenic mice studies. For the H-2D^d transgenic mice, this genetic manipulation does not alter the overall percentage of Ly-49A^+ NK cells, but instead only reduces their Ly-49A expression levels (Karlhofer et al., 1994; Olsson et al., 1995). Nevertheless, the functional phenotype of the Ly-49A^+ NK cells is clearly altered, as they preferentially lysed $D^{d\ low}$ but not the $D^{d\ high}$-expressing targets (Olsson et al., 1995; Olsson et al., 1997). Therefore, the Ly-49A expression of these Ly-49A^{low} NK cells is being calibrated so that they are readily inhibited by D^d expressed at high level, whereas target cells expressing low levels of D^d remain sensitive to this NK cell subset. In contrast, normal B6 Ly-49A^+ (Ly-49^{hi}) NK cells are prevented from lysing target cells expressing either high or low levels of D^d. These observations strongly suggest that qualitative differences in Ly-49A expression levels, and ultimately the level of D^d engaged, will determine the functional outcome of the interaction with NK cells.

In this study, we isolated Ly-49A homodimers from the T lymphoma, EL-4, of B6 origin. By using these isolated materials in *in vitro* binding assays, we further demonstrated the ligand specificity of Ly-49A and examined the effects of varying the density of Ly-49A homodimers on their interaction with H-2D^d-expressing tumors. In addition, the qualitative and quantitative aspects of these interactions in relation to receptor calibration are also discussed.

B. Materials and Methods

Cell lines. The EL-4 (H-2^b) and S49.1 (H-2^d) lymphomas were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The NZB1.1 (H-2^d) T lymphoma, Sp2/0 (H-2^d) and NS-1 (H-2^d) myelomas were kindly provided by Dr. H. Ostergaard (University of Alberta, Edmonton, AB., Canada). The A20.Cy (H-2^d) B lymphoma was a kind gift from Dr. A. O'Rouke (Scripps Clinic, La Jolla, CA). All cell lines were maintained in DMEM with 5% heat-inactivated FCS, 20 mM Hepes, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

mAbs. 34-5-8S (IgG$_{2a}$), anti-D^d (Ozato et al., 1982), SF1-1.1.1 (IgG$_{2a}$) (Roth et al., 1994) and 20-8-4S (IgG$_{2a}$) (Ozato and Sachs. 1981), anti-K^d, UPC10 (IgG$_{2a}$), anti-2-6-linked fructosan, and A1 (IgG$_{2a}$), anti-Ly-49A (Nagasawa et al., 1987) have been characterized. These hybridomas were obtained from ATCC (Manassas, VA) except A1 and UPC10 which were obtained from Dr. J. Allison (University of California Berkeley, Berkeley, CA) and Sigma Chemical Co. (St. Louis. MO), respectively. All purified mAbs were obtained by $(NH_4)_2SO_4$ precipitation of hybridoma supernatants and dialyzed against PBS. FITC-conjugated rat anti-mouse IgG was obtained from Jackson ImmunoResearch Laboratory (West Grove, PA).

Ly-49A isolation. Ly-49A molecules were isolated from the T lymphoma cell line, EL-4, grown *in vivo* as ascites in syngeneic C57BL/6 mice. In some cases, EL-4 cells were cell surface labeled with ^{125}I catalyzed by lactoperoxidase/glucose oxidase, and their lysates mixed with larger routine EL-4 extracts as described (Shen and Kane. 1995). The EL-4 cells ($1x10^{10}$) were solubilized with 0.5% NP-40, 150 mM NaCl, 10 mM Tris-HCl pH 7.6 extraction buffer and cell extracts were loaded onto Sepharose 4B precolumn followed by a CNBr-coupled A1 mAb column. The A1 column was washed extensively with 1% Triton X-100, 0.5 M NaCl, 10 mM Tris-HCl pH 7.5 and 0.1% Triton X-100, 0.15 M NaCl, 10 mM Tris-HCl, pH 8.5. Bound Ly-49A molecules were eluted from the column with 0.05% Triton X-100, 0.15 M NaCl, 100 mM glycine-HCl pH 2.9, and neutralized with 2 M Tris-HCl pH 7.5. Eluted protein was quantitated after dialysis with 0.1% n-octyl β-D glucosidase in PBS by the bicinchoninic acid assay (Pierce, Rockford, IL) or directly by gel electrophoresis with protein standard. Preparations were also standardized by solid phase ELISA with A1 mAb and stored frozen at -20^{o}C.

One- and two-dimensional gel electrophoresis of Ly-49A. Ly-49A samples were separated on 10% SDS-PAGE gels under nonreducing or reducing conditions in one dimension. Samples run in reducing conditions were boiled in 2% SDS sample buffer containing 1.44 M 2-ME, while samples for nonreducing gel runs were boiled in SDS sample buffer containing 0.05 M iodoacetamide without 2-ME. Samples were also analyzed by two-dimensional electrophoresis. The first dimension was non-equilibrium pH gradient electrophoresis (NEPHGE) using 2% Bio-Lyte 3/10 ampholyte (Bio-Rad, Mississauga, ON, Canada). NEPHGE was carried out for 2400 V h. This was followed by separation in the second dimension in 10% SDS-PAGE reducing gel (Nagasawa et al., 1987); Ly-49A was visualized by autoradiography.

Removal of N-linked oligosaccharides. The ^{125}I-labeled, purified Ly-49A protein was precipitated by cold acetone in the presence of BSA carrier (Shen and Kane. 1995). After standing at -20^{o}C overnight, precipitates were resuspended and heated for 4 min at 95^{o}C in sample buffer containing 100 mM sodium phosphate, 50 mM EDTA, 1% NP-40, 0.2% SDS and 1% 2-ME. Samples were then incubated with 2 U PNGase F (Sigma) at 37^{o}C for 20 h in the presence of 0.4 mg/ml aprotinin and 5 mM phenylmethylsulfonyl fluoride (PMSF).

Immunofluorescence staining. Half a million tumor cells were incubated with 100 µl of 34-5-8S, SF-1.1.1, or B27M1 supernatants for 30 min at 4^{o}C. Cells were extensively washed and stained with 100 µl of the 1:100 dilution of FITC-conjugated rat anti-mouse IgG. Samples were then analyzed by FACScan (Becton Dickinson, Mountain

View, CA). Cells were ranked for class I MHC allele expression by mean fluorescence intensity (MFI): (-), <5 MFI; (+/-), ≤20 MFI; (++), >40 MFI.

Cell adhesion assay. Isolated Ly-49A was immobilized on 96-well microtitre plate and confirmed by solid-phase ELISA as described (Shen and Kane. 1995). The method used to determine cell adhesion in microtitre wells bearing immobilized membrane proteins has been described in detail (Shen and Kane. 1995). Briefly, cells were labeled with $Na_2{}^{51}CrO_4$ and aliquoted at $5x10^4$ cells/well, after which plates were then centrifuged for 3 min at 400-500 rpm and incubated for 1 h, whereupon unbound cells were removed by pipeting. In some experiments, antibodies were added to the cells or the plate wells at 5 μg/ml, 20 min prior to addition of the cells to the wells. Following cell incubations on Ly-49A or control wells bearing BSA, aliquots of supernatants were harvested to determine spontaneous release. The unbound cells were removed, the well bottom cut off and radioactivity determined. Data were obtained with triplicate wells unless otherwise stated. Cell binding was calculated as percent cells bound = 100 x [(cpm bound/(total cpm - spontaneous cpm)].

C. Results

Isolation and biochemical characterization of Ly-49A. The A1 mAb is specific for the B6 Ly-49A gene product (Ly-49A^{B6}), and does not react with any other known B6 origin Ly-49 receptors (e.g. Ly-49B - I) (Takei et al., 1997), nor the Balb/c allele of Ly-49A which is 98% identical in amino acid sequence (Held et al., 1995). Detergent lysates were prepared from EL-4 tumor cells grown as ascites and passaged over an A1 immunoaffinity column. A portion of EL-4 cells were cell surface labeled with ^{125}I and mixed with unlabeled EL-4 cells, as a tracer, prior to detergent solubilization. After appropriate wash steps, bound material was eluted at pH 2.9 and neutralized. Representative samples from the A1 column were run under nonreducing and reducing conditions and visualized by autoradiography (Fig. 2-1). Ly-49A molecules are expressed at the cell surface as disulfide-linked homodimers (Yokoyama, 1995). Under nonreducing conditions, the homodimer is resolved as a single band with M_r ranges from approximately 84 kDa to 88 kDa in SDS-PAGE gel. Under reducing conditions, the homodimer migrates as a monomer with M_r ranges from approximately 42 kDa to 44 kDa (Nagasawa et al., 1987). As indicated in Fig. 2-1, the material isolated from EL-4 using the A1 column showed the predicted M_r of Ly-49A under both nonreducing and reducing conditions.

To further characterize the isolated Ly-49A, A1-reactive column eluted fractions indicated by ELISA were pooled and analyzed by two-dimensional electrophoresis. The labeled material was first subjected to separation by NEPHGE, followed by 10% SDS-PAGE. Again, a molecule of 42 kDa to 44 kDa was observed by autoradiograpghy (Fig. 2-2A) with a relatively acidic pI (5.0-5.5), consistent with that originally reported for Ly-49A (Nagasawa et al., 1987). To further confirm the identity of the isolated molecules, we treated the isolated material with PNGase F to remove *N*-linked carbohydrates, and followed by two-dimensional gel electrophoresis and autoradiography. The Ly-49A polypeptide backbone has a M_r of 31 kDa and pI of 8.8, as predicted from its amino acid sequence using MacProMass software. The normal addition of carbohydrate moieties to the three predicted *N*-linked glycosylation sites on Ly-49A during biosynthesis shifted its pI to 5.0-5.5 (Fig. 2-2A) (Nagasawa et al., 1987). Upon removal of these *N*-linked carbohydrates by PNGase F, the M_r of the isolated material was shifted from 45 kDa to 31 kDa, and the pI changed from 5.0-5.5 to 8.5-8.8 relative to other protein standards (Fig. 2-2B). This is consistent with the predicted pI values for Ly-49A. It is noteworthy that the PNGase F-treated Ly-49A appeared to be slightly more acidic than the value predicted based on its intact polypeptide backbone. This is likely due to the action of PNGase F which, upon removal of *N*-linked carbohydrates, converts carbohydrate-linked asparagines to aspartates (Tarentino and Plummer. 1994). Co-isolation of other Ly-49 members is very unlikely since the A1 mAb, as mentioned, is highly specific for the Ly-49A^{B6}.

Furthermore, the pIs of several Ly-49 members are distinct from Ly-49A and thus would have been readily distinguished in the two-dimensional gels. We therefore conclude that the A1 mAb is isolating Ly-49A homodimers from EL-4 cells.

H-2D^d expression is required for cell binding to isolated Ly-49A. Cell lines expressing various densities of H-2D^d and K^d, as determined by flow cytometry, were compared for their abilities to bind to isolated Ly-49A. Cells expressing significant levels of H-2D^d such as A20.Cy and S49.1 bound substantially to the Ly-49A immobilized on plastic (Fig. 2-3A). In contrast, Sp2/0 and NS-1, which are known to express very low levels of H-2D^d, did not bind Ly-49A significantly above background (Fig. 2-3A). Since it has been demonstrated previously that H-2D^d can serve as a ligand for Ly-49A (Kane. 1994), we tested the ability of antibodies directed against class I MHC products to block adhesion of H-2D^d-expressing cells to immobilized Ly-49A. As expected, an mAb specific for D^d, but not an isotype control or mAb directed against K^d, interfered with the adhesion of S49.1 (Fig. 2-3B) and other D^d-expressing cell lines (data not shown) to immobilized Ly-49A. Furthermore, cell adhesion was also blocked by preincubation of immobilized Ly-49A with the A1 antibody (Fig. 2-3B). These results strongly indicate that the observed cell adhesion is mediated by Ly-49A and D^d.

Relationship between Ly-49A density and tumor cell adhesion. The density of membrane protein immobilized on plastic can be varied proportionally to the input level (nanograms protein/well) over a significant density range (Shen and Kane. 1995). To examine the effect of varying Ly-49A density on cell adhesion, isolated Ly-49A was immobilized in a range between 0 - 20 ng/well input. Increases in solid-phase density with greater Ly-49A concentration were confirmed by ELISA with A1 mAb (Fig. 2-4). Adhesion of the H-2^d-expressing NZB1.1 and S49.1 cell lines to the immobilized Ly-49A at different densities was then determined in parallel (Fig. 2-4). The results indicated that the adhesion of both cell lines showed a threshold Ly-49A density dependence of 3 - 5 ng/well input, which was at a density above the minimum detectable by A1 ELISA. Above this threshold, the extent of cell adhesion closely paralleled the increases of Ly-49A density. Cell binding and ELISA begin to plateau at 10 - 20 ng/well input, which is probably due to saturation of Ly-49A or detergent interference with Ly-49A immobilization at the higher protein input levels (Kane et al., 1989). Substantial changes in the cell binding occur over a twofold density range and is reminiscent of the narrow class I MHC density dependence of CTL CD8-mediated adhesion (Shen and Kane. 1995). The demonstration that nanogram input concentrations of Ly-49A per well support the cell adhesion suggests that very low densities of Ly-49A are mediating class I MHC-dependent cell adhesion, and thus the avidity of Ly-49A interaction with H-2D^d is likely to be relatively high.

D. Discussion

Ly-49A density requirements for cell adhesion. Individual murine NK cells are known to simultaneously express multiple Ly-49 family members at the cell surface (Brennan et al., 1994; Held et al., 1995). This greatly complicates efforts to define class I MHC specificities, to characterize the nature of interaction, and to attribute functional outcomes to specific Ly-49 receptors. Therefore, in order to examine Ly-49A and D^d interactions in more detail, we isolated Ly-49A from the EL-4 lymphoma, which expresses high levels of this receptor (Nagasawa et al., 1987). The isolated molecule behaves as a homodimer, retains its conformational integrity, and mediates H-2D^d-dependent cell adhesion when immobilized on plastic.

By varying the input concentration of Ly-49A in the plate wells, the density of Ly-49A was varied accordingly, and this was confirmed by solid-phase ELISA. Generally, approximately 30% of the input amounts of membrane proteins and soluble proteins bind plastic microtitre wells at concentration below saturation (Butler et al., 1987). Therefore, from the input concentration of isolated Ly-49A and the size of the well, it can be estimated from our data that for the two D^d-expressing tumor cells NZB1.1 and S49.1 to achieve half-maximal binding, a Ly-49A density of 210 - 285 dimers/μm^2 is required, whereas a homodimer density of 140 dimers/μm^2 is needed in order to detect cell binding above background. These densities are quite low and comparable to the densities of immobilized LFA-1 integrin required to observe ICAM-1 dependent cell adhesion (Dustin and Springer. 1989). The density of Ly-49A on NK cells has not been quantitated, although it is expected to be fairly low from flow cytometry analysis (Daniels et al., 1994a). This is especially true for those Ly-49A$^+$ NK cells derived from H-2^d mice. If we were to extrapolate our results to NK cells by assuming a cell of 5 μm in diameter, a density of about 2800 Ly-49A dimer per cell could be the minimum Ly-49A cell surface density to detect class I MHC-dependent cell binding. The density required to achieve half-maximal cell binding would translate to about 4900 dimers per cell surface. Such Ly-49A cell surface densities are relatively low and may be within the range expressed on Ly-49A$^+$ NK cells. Our observation that small numbers of immobilized Ly-49A molecules support adhesion of D^d-expressing cell suggests that the interaction between the cells and the plate surface is of a relatively high avidity. It is perhaps noteworthy in this context that the density of Ly-49A required to observe mouse class I MHC-dependent cell adhesion reported here is 100-fold less than the probable density of a recombinant Ig fusion protein of NKAT2 (a KIR specific for HLA-Cw1) immobilized on solid phase required to observe HLA-Cw1-dependent cell adhesion (Dohring and Colonna. 1996). Such density requirement differences could suggest that the mouse Ly-49A molecule has a higher affinity/avidity for a class I MHC ligand than a human receptor of similar function; however further studies are necessary to directly address this possibility.

Ly-49A density and receptor calibration model. Olsson and colleagues have shown that the expression of D^d as a transgene in B6 mice leads to reduction of Ly-

49A expression, and alters the functional phenotype of these Ly-49A$^+$ NK cells (Olsson et al., 1995). As a result of this reduction, these cells are not inhibited by a low level of D^d expression, and more sensitive to small changes in D^d expression levels of the target cells. It is assumed that the failure to protect target cells expressing low levels of D^d is due to insufficient engagement of Ly-49A and D^d, which falls below a minimum threshold for adequate signal transduction. In this study, we demonstrated that there is indeed a threshold Ly-49A density required for detectable D^d-dependent cell adhesion to occur. This is followed by a rapid increase in cell adhesion as the Ly-49A density increases above this threshold. Therefore, our results are consistent with the receptor calibration model (Olsson et al., 1995; Olsson et al., 1997), and demonstrated that over a narrow range of Ly-49A density profound differences in the degree of D^d engagement are observed.

Our *in vitro* binding assay provides direct evidence that small differences in Ly-49A density can significantly affect the extent of stable Ly-49A receptor engagement. However, the Ly-49 receptor calibration model is proposed to be a general phenomenon and extended to other Ly-49 receptors. Whether other Ly-49 receptors also exhibit similar threshold density dependent cell adhesion as seen with Ly-49A remains to be investigated.

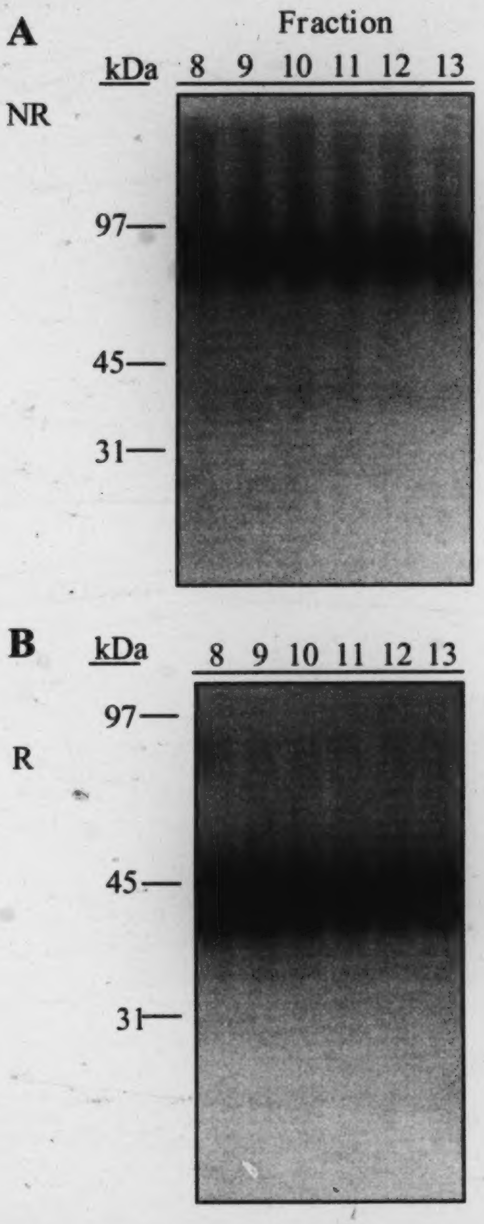

A
Fraction
kDa
8 9 10 11 12 13
NR
97
45
31
B
kDa
8 9 10 11 12 13
97
R
45
31

Figure 2-1. Nonreducing and reducing SDS-PAGE gels of isolated Ly-49A. Detergent lysates from 10^{10} EL-4 cells were applied to an A1 mAb affinity column and A1-reactive materials were eluted as described in *Materials and Methods.* Representatives A1 column samples (30 µl) were analyzed by SDS-PAGE on 10% gels under nonreducing (A) and reducing conditions (B) and autoradiographed.

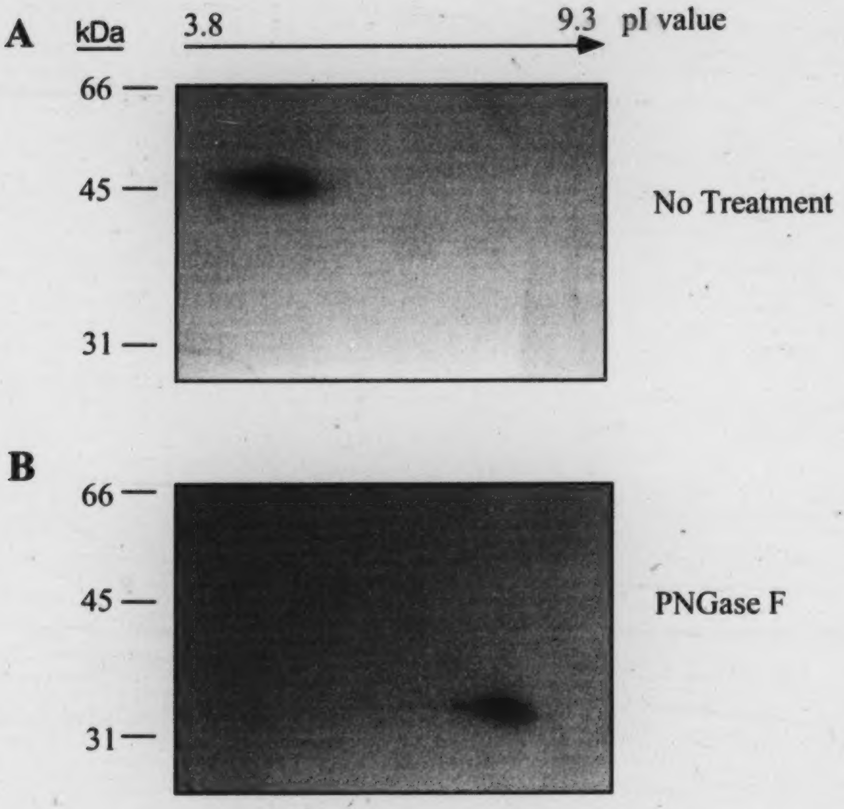

A
kDa
3.8
9.3 pI value
66
45
31
No Treatment
B
66
45
31
PNGase F

Figure 2-2. PNGase F treatment and two-dimensional electrophoresis of Ly-49A. Samples of Ly-49A (30 µl) were mock-treated (A) or with 2 U PNGase F (B). Samples were run on NEPHGE in the first dimension (2400 V-h) with gel containing 2% Bio-lyte 3/10 ampholyte, followed by 10% SDS-PAGE under reducing conditions and visualized by autoradiography.

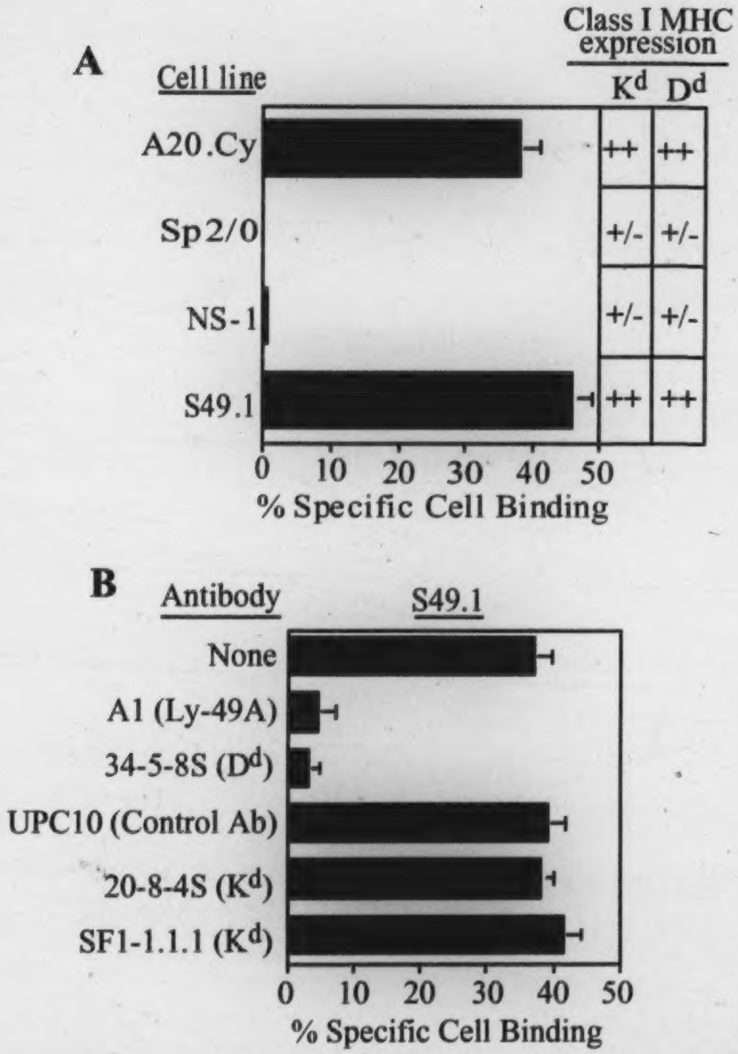


Figure 2-3. Cell adhesion to immobilized Ly-49A is dependent on H-2D^d. ^{51}Cr-labeled tumor cells (5x10^4) were added directly (A) or pre-incubated alone or with 5 μg/ml antibody or isotype control mAb (B) and incubated for 1 h on immobilized Ly-49A (8 ng) at 37^{o}C. In the case of A1 mAb, plate-bound Ly-49A was pre-incubated with this mAb before addition of tumor cells. Unbound cells were removed and the percent cell binding was determined. The relative expression level of class I MHC alleles is indicated by plus and minus designations, (++) high, (+/-) very low, or (-) none. Cell binding is expressed as the mean percent ± SD of triplicate wells minus BSA background binding (< 5%).

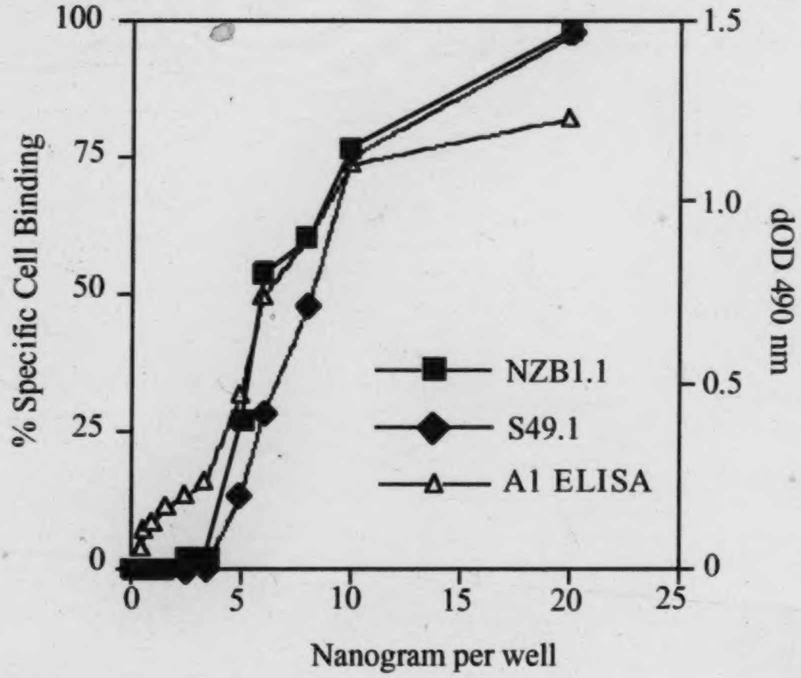


Figure 2-4. Density requirements of isolated Ly-49A for adhesion by H-2D^{d}-expressing cells. ELISA reactivity with A1 primary antibody and horseradish peroxidase-coupled goat anti-mouse secondary antibody was determined at 490 nm for Ly-49A immobilized at the indicated densities (open triangle). Percent specific cell binding was determined with S49.1 cells (solid diamond) and NZB1.1 cells (solid square) after 1 h of incubation at 37°C on Ly-49A coated wells. Results are shown as mean specific binding of triplicate wells after substraction of nonspecific binding to BSA (< 5%).

CHAPTER III

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EVIDENCE FOR SULFATE MODIFICATION OF H-2D^d ON *N*-LINKED CARBOHYDRATE(S): POSSIBLE INVOLVEMENT IN Ly-49A INTERACTION

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A. Introduction

A distinctive characteristic of class I MHC molecules is their extreme polymorphic polypeptide backbones. In addition, each class I MHC allelic product also possesses substantial microheterogeneity primarily attributed to the variable size, branching and charge of the *N*-linked carbohydrate moieties (Sweidler et al., 1983; Krakauer et al., 1980; Landolfi and Cook. 1986). All of the murine classical class I MHC molecules have two conserved *N*-linked glycosylation sites on residues N86 and N176, which are located at the α1 and α2 domains, respectively (Watts et al., 1989). Some murine class I MHC molecules have an additional *N*-linked glycosylation site in the α3 domain (Watts et al., 1989). In contrast, only a single *N*-linked glycosylation site is found on human class I MHC molecules. Furthermore, human class I MHC *N*-linked carbohydrate moieties appear to have a more uniform structure (Baber et al., 1996).

The *N*-linked carbohydrates of class I MHC molecules are known to be not required for CTL-target cell recognition (Goldstein and Mescher. 1985; Miyazaki et al., 1986). In fact, certain *N*-linked carbohydrate modifications on class I MHC molecules may actually interfere with class I MHC-restricted TCR interactions (Shen and Kane. 1995). Therefore, besides involvement in the class I MHC assembly pathway, no other function has been ascribed to the class I MHC *N*-linked carbohydrates. However, with the recent description of various animal C-type lectin NK cell regulatory receptors (Lanier. 1998b), the importance of these *N*-linked carbohydrates in the class I MHC regulation of NK cell functions has been a major focus of debate.

Among all of the NK cell inhibitory receptors, Ly-49A has been used as the primary model to test the hypothesis that its CRD can interact with carbohydrate structures. To this end, Seaman and colleagues have provided the first direct evidence which showed that CRD of Ly-49A is indeed functional and able to bind sulfated-fucose containing polysaccharide such as fucoidan (Daniels et al., 1994b). Furthermore, the cytotoxic activity of Ly-49A$^+$ NK cells against H-2D^d-expressing targets can be enhanced in the presence of sulfated monosaccharides or by treating the target cells with the *N*-linked glycosylation inhibitor tunicamycin (Daniels et al., 1994b). This implies that Ly-49A has to interact with some form of carbohydrate structures on the target cells in order to deliver inhibitory signals.

Not surprisingly, the ability to bind sulfated carbohydrates is not confined to Ly-49A. For example, the binding of an H-2^s-expressing cell line to Ly-49C transfected COS cells was also inhibited by sulfated polysaccharides including fucoidan and λ-carrageenan (Brennan et al., 1995). More importantly, it was shown that this interaction could be eliminated by treating target cells with fucosidase (Brennan et al., 1995), strongly suggesting that Ly-49C is capable of recognizing specific carbohydrate structures. The involvement of the Ly-49 CRD in ligand interaction is demonstrated through domain swapping experiments. For instance, the interaction between Ly-49C and H-2^b and H-2^s-expressing cells involves the CRD and the 19 amino acids adjacent to this region (Brennan et al., 1996b). Therefore, all of these results support the notion that carbohydrate moieties are critical in the interaction of Ly-49 receptors and their class I MHC ligands.

Since D^d is a known cognate class I MHC ligand for Ly-49A (Kane. 1994; Daniels et al., 1994a), and the Ly-49A CRD binds sulfated polysaccharides, this raises the possibility that the carbohydrate moieties on D^d could be sulfated. However, to date, class I MHC molecules have not been reported to undergo the process of sulfation. In this study, we examined whether D^d isolated from primary spleen cell culture and from a variety of tumor cell lines could be sulfated on its *N*-linked carbohydrates. In addition, the effect of this post-translational modification on the interaction of D^d and Ly-49A was also investigated.

B. Materials and Methods

Mice. Eight to twelve-week old female Balb/c mice were obtained from the University of Alberta mouse breeding facility (Edmonton, AB., Canada).

Enzymes and Chemicals. Endoglycosidase F (Endo F)/peptide *N*-glycosidase F (PNGase F) and PNGase F purified from *Flavobacterium meningosepticum* were purchased from Boehringer Mannheim (Laval, PQ) and Sigma Immunochemicals (St. Louis, MO), respectively. Recombinant *Streptomyces plicatus* endoglycosidase H (Endo H) was purchased from Boehringer Mannheim (Laval, PQ). Concanavalin A (Con A) was from Pharmacia Fine Chemicals (Uppsala, Sweden). Immobilized protein A on Trisacryl GF-2000 was obtained from BioRad (Rockford, IL). Fischer's sulfate-free medium, sulfate-free DMEM, and RPMI-1640 Select-Amine® Kits were obtained from Gibco BRL (Burlington, ON). Autoradiography enhancer, EN^3HANCE, was purchased from DuPont (NEN, Richmond, B.C.). Tunicamycin and BSA (Fraction V) were purchased from Sigma Immunochemicals (St. Louis, MO). Sodium chlorate ($NaClO_3$) was purchased from Fisher Scientific (Fair Lawn, NJ). $Na_2{}^{35}SO_4$ in water (~43 Ci/mg S) and $Tran^{35}S$ label (>1000 Ci/mmol) were purchased from ICN Pharmaceuticals (Irvine, CA).

mAbs. 34-5-8S (IgG_{2a}), anti-D^d (Ozato et al., 1982), 11-4.1 (IgG_{2a}), anti-K^k (Oi et al., 1978), B22.249 (IgG_{2a}), anti-D^b (Lemke et al., 1979), AF6-88.5 (IgG_{2a}), anti-K^b (Loken and Stall. 1982) and A1 (IgG_{2a}), anti-Ly-49A (Nagasawa et al., 1987) have been characterized. These hybridomas were obtained from ATCC (Manassas, VA) except A1 and B22.249 which were obtained from Dr. J. Allison (University of California Berkeley, Berkeley, CA) and from Dr. U. Hammerling (Memorial Sloan-Kettering Cancer Center, New York, NY), respectively. Purified mAbs were obtained by $(NH_4)_2SO_4$ precipitation of hybridoma supernatants and dialyzed against PBS. FITC-conjugated rat anti-mouse IgG was obtained from Jackson ImmunoResearch Laboratory (West Grove, PA).

Tumor Cell Lines. The S49.1 and Yac-1 T lymphomas were obtained from ATCC (Manassas, VA). The A20.Cy B lymphoma and NZB1.1 T lymphoma were provided by Dr. A. O'Rourke (Scripps Clinic, La Jolla, CA) and Dr. H. Ostergaard (University of Alberta, Edmonton, AB., Canada), respectively. The A20.Cy, S49.1 and NZB1.1 cell lines were maintained in DMEM with 5% heat-inactivated FCS, 20 mM Hepes, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The Yac-1 was grown in RPMI with the same supplements as in DMEM.

Immunofluorescence staining. Half a million cells were stained with the indicated mAbs for 30 min and washed, then counter stained with 100 μl of 1:100 dilution of FITC-labeled rat anti-mouse IgG for the same length of time. The whole staining procedure was carried out at 4°C.

Isolation of resting T cells and activation of T cells by Con A. To isolate resting T cells, Balb/c splenocytes were depleted of RBCs by 0.14 M NH_4Cl in Tris, pH 7.3, and then incubated at $5x10^6$/ml on tissue culture-treated plates for an hour at 37°C in DMEM/10% FCS. The plastic non-adherent cells were harvested and further passaged, at a density of 1×10^7 cells/ml, through a nylon wool column. The unbound cells were then used for radiolabeling. To activate T cells by Con A, RBC-depleted splenocytes were cultured in DMEM/10% FCS with 3 μg/ml of Con A in a 24-well plates at 5×10^6/ml. After 48 h of culture, the activated cells were harvested and used for radiolabeling.

Radiolabeling. Cells were metabolically labeled at 2×10^6 cells/ml for 7 to 8 h at 37°C. For sodium ^{35}sulfate (SO_4^{-2}) labeling, cells were incubated with 1 mCi/ml of $Na_2{}^{35}SO_4$, in Fischer's medium or sulfate-free DMEM supplemented with 10% dialyzed FCS. For Tran^{35}S (^{35}S methionine and ^{35}S cysteine) labeling, cells were incubated with 200 μCi/ml of radioactive label in methionine-free RPMI with 10% dialyzed FCS.

Inhibition of D^d sulfation by sodium chlorate and N-linked glycosylation by tunicamycin. Cells at a concentration of 2×10^6/ml were labeled with $Na_2{}^{35}SO_4$ in Fischer's medium supplemented with 10% dialyzed FCS for 7 h, in the presence of 10 or 20 mM of $NaClO_3$. To inhibit *N*-linked glycosylation, cells were labeled with $Na_2{}^{35}SO_4$ or Tran^{35}S label in Fischer's medium or methionine-free RPMI supplemented with 10% dialyzed FCS in the presence of 1 μg/ml of tunicamycin for 7 h.

Immunoprecipitation, SDS-PAGE and autoradiography. Five to ten million metabolically labeled cells were lysed at 1×10^7 cells/ml in PBS containing 1% NP-40, 1 mM PMSF and 5 mM iodoacetamide, pH 7.2, at 4°C. Cell lysates were centrifuged at 16000*g* for 15 min and supernatants collected. The lysate was precleared twice with 30 μl of protein A packed beads and 5 μl of Balb/c normal serum, followed by immunoprecipitation with 30 μl of protein A packed beads and 10 μg of 11-4.1 or 34-5-8S mAb per 10^7 cells for 2 h. Beads were washed with lysis buffer containing 1% deoxycholate, pH 7.2, and eluted with 60 μl of 2% SDS reducing sample buffer, at 100°C for 4 min. The eluted materials were resolved by 10% SDS-PAGE under reducing conditions (Laemmli. 1970). After intensification by autoradiography enhancer (EN^{3}HANCE), the gels were vacuum-dried and exposed to XAR5 Kodak film.

Two-dimensional gel electrophoresis of radiolabeled class I MHC. In some experiments, $^{35}SO_4^{-2}$ and Tran^{35}S labeled D^d were eluted from mAb bearing beads and subsequently acetone precipitated with 5 μg of BSA carrier to remove 2-ME prior to 2-D gel electrophoresis. Precipitates were then resuspended in isoelectric focusing (IEF) sample buffer and separated by IEF for 4800 V-h using a 4 to 1 ratio of Bio-Lyte 5/7 and Bio-Lyte 3/10 (O'Farrell. 1975). The second dimension separation was then carried out in 10% SDS-PAGE reducing gels.

Enzymatic removal of N-linked carbohydrates. Eluted radiolabeled proteins from antibody bearing beads were alkylated as described (Sweidler et al., 1983), and acetone precipitated at -20°C overnight with BSA carrier. For Endo F/PNGase F digestion, acetone precipitates were resuspended and heated for 4 min at 100°C in 50 μl sample buffer containing 80 mM sodium phosphate, 50 mM EDTA, 1% NP-40, 0.2% SDS and 1% 2-ME, pH 6.0. For Endo H treatment, the buffer was 0.1M sodium acetate, 0.2% SDS, 1% 2-ME, pH 6.0. Samples were then digested by 0.2 mU EndoF/PNGase F, 2 U PNGase F or 2 mU of Endo H at 37°C for 10 h in the presence of 0.2 mg/ml aprotinin and 2 mM PMSF. After 10 h of incubation, half of the original amount of enzyme was added again and the digestion was carried out for another 10 h.

Cell adhesion assay. Ly-49A isolation and cell adhesion assay using isolated Ly-49A have been described previously (Chapter II; Chang et al., 1996). Briefly, 5x10^4 Na^{51}CrO$_4$-labeled cells were added to each well of microtiter plates immobilized with isolated Ly-49A at various densities or a control protein BSA, following preincubation for 20 min with medium or antibodies at 5 μg/ml. The plates were centrifuged at 400 rpm for

5 min and incubated at 37°C for 1 h. The unbound cells were removed and the percentage of bound cells was calculated as [cpm bound/(total cpm -spontaneous cpm)] x 100. For carbohydrate inhibition assay, polysaccharides were added to each well to give the indicated final concentrations.

C. Results

Inhibition of H-2D^d-expressing tumor binding to immobilized Ly-49A by sulfated polysaccharides. We have previously demonstrated that adhesion of cells of H-2^d origin to isolated Ly-49A can be blocked by anti-D^d and anti-Ly-49A mAbs 34-5-8S and A1, respectively (Chapter II; Chang et al., 1996). A similar observation was seen in the binding of NZB1.1 to immobilized Ly-49A (Fig. 3-1), showing that the cell binding is indeed mediated by Ly-49A and D^d. Since sulfated mono- and polysaccharides have been shown to interfere with the interaction of D^d and Ly-49A in cell-cell binding assays (Daniels et al., 1994b), we thus attempted to examine this phenomenon by using the *in vitro* cell adhesion system. In agreement with the previously published data (Daniels et al., 1994b; Brennan et al., 1995), certain soluble sulfated sugar structures were capable of preventing NZB1.1 from binding to isolated Ly-49A immobilized on a plastic surface (Fig. 3-2). No inhibition of NZB1.1 binding to isolated Ly-49A was observed in the presence of negatively charged hyaluronic acid or neutral charged mannan. In contrast, inhibition of NZB1.1 binding to Ly-49A was detected in the presence of sulfated polysaccharides. Interestingly, not all of the sulfated polysaccharides interfered with this interaction. Among the sulfated polysaccharides tested, fucoidan and heparin possessed the greatest inhibitory abilities, followed by chondroitin-4-sulfate. In contrast, chondroitin-6-sulfate exhibited no inhibitory effect even at high concentrations (Fig. 3-2). Therefore, these data suggest that Ly-49A is capable of recognizing sulfated polysaccharides. Furthermore, Ly-49A appears to preferentially interact with certain sulfated carbohydrate structures over others.

Isolation of sulfated class I MHC D^d from various tumor cell lines. Since the data from carbohydrate inhibition assay suggested that Ly-49A is capable of binding sulfated carbohydrate moieties, we decided to investigate the possibility that D^d molecules themselves display sulfate moieties that could perhaps explain the binding competition offered by soluble sulfated polysaccharides. To this end, NZB1.1 was labeled with $^{35}SO_4^{-2}$ or Tran^{35}S label for 7-8 h in Fischer's sulfate-free medium and Met-free RPMI, respectively. As expected, the 45 kDa D^d heavy chain and the 12 kDa β_2m light chain were immunoprecipitated from the Tran^{35}S-labeled cell lysate by the mAb 34-5-8S, but not the isotype control mAb, 11-4.1 (Fig. 3-3A, lanes 1 and 2). Interestingly, the autoradiograph also indicated that a sulfated form of the D^d heavy chain was immunoprecipitated from the $^{35}SO_4^{-2}$-labeled cell lysate using the same D^d-specific mAb, whereas no sulfo-labeled band was immunoprecipitated using the isotype control mAb, 11-4.1 (Fig. 3-3A, lanes 3 and 4). Sulfated D^d was also detected in cells labeled in sulfate-free DMEM (Fig. 3-3B, lanes 1 and 2). Therefore, sulfation of D^d was not specific for cells that have been labeled in Fischer's medium. In addition, no change in D^d expression was detected in cells that had been cultured in Fischer's medium as indicated by FACS analysis (Table 3-1). These results strongly indicate that NZB1.1 expresses sulfated forms of H-2D^d.

Since D^d isolated from NZB1.1 was sulfated, this post-translational modification was further investigated in other H-2D^d-expressing tumor cell lines. For all cell lines tested, A20.Cy, S49.1 and Yac-1, sulfated D^d could be detected by immunoprecipitation (Fig. 3-4, lanes 6,7 and 8). However, it is important to note that not all D^d are sulfated to the same extent and that sulfation bears no correlation with the D^d expression level. For instance, when comparing the Tran^{35}S- and $^{35}SO_4^{-2}$-labeled immunoprecipitates, A20.Cy and S49.1 incorporated substantial Tran^{35}S label (Fig. 3-4, lanes 2 and 3), but yielded the least amount of $^{35}SO_4^{-2}$-labeled D^d (Fig. 3-4, lanes 6 and 7). In contrast, NZB1.1 and Yac-1 which have lower levels of D^d cell surface expression and thus incorporated Tran^{35}S label to a lesser extent (Fig. 3-4, lanes 1 and 4), have higher amounts of sulfated D^d (Fig. 3-4, lanes 5 and 8). In addition, within the M_r ranges of the D^d observed by Tran^{35}S labeling for each cell line, the M_r of the sulfated D^d species differ among cell lines. This is probably due to *N*-linked glycosylation microheterogeneity and sulfation of specific D^d glycoforms expressed by these cell lines (Fig. 3-4). For example, S49.1 has sulfated D^d of the highest M_r among all of the tumors cell lines analyzed (Fig. 3-4, lane 7), with a corresponding portion of the S49.1 Tran^{35}S-labeled D^d having an identical M_r (Fig. 3-4, lane 3).

Sulfo-labeling of resting T-cells and Con A-activated T-cells. Since all D^d-expressing tumor cells that were examined expressed sulfated forms of D^d, we wished to determine whether D^d expressed by normal and Con A-activated T cells were also sulfated. Normal resting T cells expressed a lower level of D^d compared to their Con A-activated counterparts and incorporate labeled amino acids at a substantially reduced rate during the labeling period. Only a very small amount of Tran^{35}S label was incorporated in the D^d immunoprecipitated from resting Balb/c splenic T cells, and no $^{35}SO_4^{-2}$-labeled D^d was detected (Fig. 3-5, lanes 2 and 6). It is still possible, however, that D^d expressed in normal resting cells does incorporate some sulfate, but the low metabolic rate of these cells does not allow us to resolve this issue with radiolabeled sulfate. In contrast, a strong incorporation of Tran^{35}S label and a low but detectable incorporation of $^{35}SO_4^{-2}$ were observed in D^d immunoprecipitated from Con A-activated T cells (Fig. 3-5, lanes 4 and 8). Thus, sulfation of the D^d molecule occurs in normal metabolically active cells and is thus not restricted to transformed cell lines.

All glycoforms of D^d isolated from NZB1.1 are sulfated. Due to the variable action of glycosyltransferases in processing *N*-linked carbohydrates, murine class I MHC molecules are known to possess a high degree of microheterogeneity (Sweidler et al., 1983; Krakauer et al., 1980; Landolfi and Cook. 1986). Therefore, to identify how many D^d glycoforms undergo sulfation, two-dimensional gel electrophoresis was carried out on D^d immunoprecipitated from NZB1.1 cells that had been labeled for 8 h with either Tran^{35}S or $^{35}SO_4^{-2}$. The radio-labeled D^d were then separated into various subpopulations by IEF followed by 10% SDS-PAGE, with isoelectric points (pIs) ranging from 5.5-6 (Fig. 3-6). The detected pI range of the separated D^d glycoforms is in agreement with that published previously (Landolfi and Cook. 1986). In addition, the pattern of separation detected with $^{35}SO_4^{-2}$-labeled D^d (Fig. 3-6B) corresponded to the mature glycoform species of D^d (toward the acidic end of the gel) observed by Tran^{35}S label (Fig. 3-6A). This strongly suggests that all of the detectable mature glycosylation variants of D^d expressed by NZB1.1 are modified with sulfate.

Sulfation of D^d occurs on N-linked carbohydrate(s). Sulfation is a post-translational modification which can occur either on tyrosine residues or carbohydrate moieties (Huttner. 1984; Roux et al., 1988). Since there are three potential tyrosine sulfation sites (Rosenquist and Nicholas. 1993) and two known *N*-linked glycosylation sites on D^d, the polypeptide backbone, the *N*-linked carbohydrates or both, may be modified with sulfate. To distinguish these possibilities, NZB1.1 cells were labeled with Tran^{35}S and $^{35}SO_4^{-2}$ in the presence of tunicamycin, an *N*-linked glycosylation inhibitor (Elbein. 1987). The "carbohydrate-less" D^d polypeptide backbone was detected in the Tran^{35}S-labeled D^d immunoprecipitate as a faint band (Fig. 3-7, lane 1 arrow). The immunoprecipitated D^d was labeled rather poorly, possibly due to the generally rapid turnover of carbohydrate-less class I MHC molecules, which are deficient in association with the ER chaperon calnexin (Ware et al., 1995). In the presence of this inhibitor, no radiolabeled material was immunoprecipitated from the $^{35}SO_4^{-2}$ labeled NZB1.1 (Fig. 3-7, lane 5). This result might imply that $^{35}SO_4^{-2}$ is added to the *N*-linked carbohydrate moieties on D^d. However, it is possible to argue that in the presence of this inhibitor, D^d

does not efficiently transit through the *trans*-Golgi network, where sulfation is thought to occur (Rosa et al., 1995). As a result, both tyrosine as well as carbohydrate sulfation simply cannot take place under the circumstance of tunicamycin treatment. To address the issue of sulfation of *N*-linked carbohydrates on D^d more directly, D^d was immunoprecipitated from Tran^{35}S and $^{35}SO_4^{-2}$-labeled NZB1.1 and treated with a mixture of Endo F and PNGase F, or PNGase F alone. Upon digestion with these enzymes, which remove *N*-linked carbohydrates, the $^{35}SO_4^{-2}$ label on D^d was no longer detectable by autoradiography (Fig. 3-7, lanes 7 and 8), in contrast to the untreated control (Fig. 3-7, lane 6). The disappearance of the sulfate label was due to removal of the *N*-linked carbohydrates since the migration of the Tran^{35}S-labeled D^d treated in parallel with the same *N*-glycosidases shifted to a position consistent with the carbohydrates-less D^d polypeptide backbone (Fig. 3-7, lanes 3 and 4). An additional fainter band with slightly reduced M_r was also observed in the untreated Tran^{35}S-labeled control (Fig. 3-7, lane 2) and following Endo F/PNGase F treatment of Tran^{35}S-labeled D^d immunoprecipitates (Fig. 3-7, lanes 3 and 4). This probably represents the protein product of a D^d mRNA splice variant that lacks thirteen amino acids in its cytoplasmic tail (McCluskey et al., 1986). Assuming this is the case, it too may be sulfated on *N*-linked carbohydrate(s) and observed as the fainter sulfated band just below the major sulfated D^d band (Fig. 3-7, lanes 6). Sulfation of this presumably differentially spliced form of D^d is supported by the observation that its $^{35}SO_4^{-2}$ label was also removed upon similar enzymatic treatments (Fig. 3-7, lanes 7 and 8). Taken together, these data indicate that the *N*-linked carbohydrate(s) on D^d expressed on NZB1.1 cells is sulfated; yet the tyrosine residues of D^d are not detectably sulfated. When $^{35}SO_4^{-2}$-labeled D^d was treated with Endo H, an endoglycosidase that removes immature high mannose content *N*-linked carbohydrates (Tarentino and Plummer. 1994), it was found to be resistant to this enzymatic treatment (Fig 3-8, lanes 1 and 2). Thus, sulfation of D^d occurs on mature (post-medial Golgi) *N*-linked carbohydrate moieties.

$NaClO_3$ inhibits sulfation of D^d and binding of NZB1.1 to immobilized Ly-49A. Since we demonstrated that sulfation of D^d on NZB1.1 cells was located on *N*-linked carbohydrates, this sulfation process should be inhibited by $NaClO_3$. This compound is a potent inhibitor of the formation of 3'-phosphoadenosine 5'-phosphosulfate (PAPS), the active *in vivo* sulfate donor (Elbein. 1987). When sulfo-radiolabeling of NZB1.1 was conducted in the presence of $NaClO_3$, sulfation of D^d was no longer detectable (Fig. 3-9, lanes 2 and 3), in contrast to the untreated control (Fig. 3-9, lane 1). The failure to incorporate $^{35}SO_4^{-2}$ was not due to the inhibition of D^d expression, since cell surface expression of D^d on NZB1.1 exposed to 20 mM $NaClO_3$ remained the same as the untreated control as detected by FACS analysis (Table 3-1). In addition, $NaClO_3$ had no effect on D^d synthesis, as comparable amount of Tran^{35}S-labeled D^d could be immunoprecipitated from both $NaClO_3$-treated and untreated NZB1.1 (data not shown). Therefore, these data indicate that chlorate ions can inhibit the sulfation of D^d *N*-linked carbohydrates.

Since certain soluble sulfated polysaccharides inhibit the binding of NZB1.1 to immobilized Ly-49A (Fig. 3-1), and the *N*-linked carbohydrate moieties on D^d are sulfated, we reasoned that pre-treatment of the D^d-expressing cells with chlorate ions should inhibit cell adhesion to Ly-49A. When NZB1.1 was cultured in sulfate-free medium, there was a slight reduction in its binding to Ly-49A relative to when they were grown in the same medium supplemented with sulfate ions (Fig. 3-10). The substantial residual binding under these conditions could be due to long-live intracellular pool of SO_4^{-2} still available to the cells for modification of D^d. In addition, the cells might also able to derive free sulfate from sulfur containing amino acids (Keller and Keller. 1987; Humphries et al., 1988), and therefore sulfation of D^d still can take place even under these culturing conditions. Furthermore, it is possible that sulfated D^d might have a longer half-life than their non-sulfated counterparts, and much of the sulfated D^d would still remain on the cell surface after culturing in sulfate-free medium. Perhaps as a combination of these factors, culturing of NZB1.1 in sulfate-free medium alone is insufficient to inhibit its binding to isolated Ly-49A. However, when these cells were treated with 20 mM $NaClO_3$ for 7 h, there was a significant reduction in the percentage of cells bound to Ly-49A (Fig. 3-10). The reduction in adhesion became particularly apparent when the density of the immobilized Ly-49A was limiting (Fig. 3-10), whereas at a high density of Ly-49A, the chlorate-treated cells were still able to interact with immobilized Ly-49A. These results suggest that sulfate modified *N*-linked carbohydrates on D^d can participate in the interaction with Ly-49, with sulfation becoming essential for cell adhesion when the Ly-49A density is relatively low.

D. Discussion

Tumor cell lines and primary cells express varying degree of sulfated glycoform of H-2D^d. We have demonstrated in this study that murine H-2D^d can be modified with sulfate. Since mammalian cells do not reduce SO_4^{-2} to sulfur-containing amino acids (Cooper. 1983), the sulfation of D^d must be a post-translational process. Sulfation can occur on tyrosine residues or on carbohydrate moieties (Huttner. 1984; Roux et al., 1988). Based on the known tyrosine sulfation consensus sequences (Rosenquist et al., 1993), D^d may have up to three potential tyrosine sulfation sites at residues 59, 118, and 123, located in the $\alpha 1$ and $\alpha 2$ domains. In addition, it has two conserved *N*-linked glycosylation sites at residues N86 and N176. After treating with PNGase F, the sulfate label of the NZB1.1 D^d was removed and no residual sulfo-radiolabel was detected on the polypeptide backbone. This strongly suggests that *N*-linked carbohydrates on NZB1.1 D^d are sulfated. However, we cannot rule out the possibility that some tyrosine sulfation might occur to a small extent, and that this sulfation might not be detected by methods employed in this study. In addition, it remains possible that D^d expressed by other tumor cell lines are sulfated on tyrosine residues and/or *N*-linked carbohydrate(s).

We observed that D^d expressed by various tumor cell is apparently not sulfated to the same extent. Among the tumor cell lines analyzed, NZB1.1, a spontaneous tumor originated from NZB mice, has the highest level of sulfated D^d in absolute terms relative to the amount of D^d expressed by these cells. In contrast, A20.Cy and S49.1 have the lowest relative sulfation of D^d, by comparing $^{35}SO_4^{-2}$-labeled D^d with Tran^{35}S-labeled D^d in immunoprecipitates and the amount of D^d expressed at the cell surface. Thus, the degree of sulfation is not directly correlated with the level of D^d expression. This difference might be accounted for by the origin, growth, and potential *in vivo* survival selection of the individual tumor cell line. The tumors may differ in the level of expression of sulfotransferases involved in the transfer of sulfate to glycoprotein acceptor molecules, or some tumor cell lines may have a larger intracellular pool of sulfate, obtain sulfate from sulfur-containing amino acids more efficiently than others (Keller and Keller. 1987; Humphries et al., 1988), or generate more or less PAPS, the active sulfate donor. In addition, the sulfated D^d immunoprecipitated from a variety of tumors can differ slightly in their relative mobilities. These differences are likely to be attributable to the variable complexity of *N*-linked carbohydrates that can be expressed on D^d (Krakauer et al., 1980). Whether other class I MHC allelic products can be sulfate modified similar to D^d remains to be determined.

Sulfation of D^d is not a phenomenon restricted to tumor cell lines, since we found that D^d immunoprecipitated from Con A-activated T cells is also sulfated. However, all of the tumor cells we have tested appeared to have a much higher degree of D^d sulfation compared to the Con A-activated T cells. Perhaps transformation can lead to overexpression of sulfotransferases or other factors which favor hypersulfation of D^d. It remains to be determined whether increase sulfation of D^d or other class I MHC molecules might provide a means for some tumor cells to escape destruction by NK cells, possibly by enhancing interaction with NK cell inhibitory receptors such as Ly-49 family members.

Two-dimensional SDS-PAGE analysis of NZB1.1 D^d indicated that sulfated forms of D^d were found to co-migrate with all major mature glycosylation variants, with pI range of 5.5 to 6.0. Therefore, sulfation may contribute to microheterogeneity of class I MHC molecules. This possibility is supported by the fact that neuraminidase treatment of D^d only partially reduces its isoelectric heterogeneity (Krakauer et al., 1980). Whether all mature glycoforms of D^d are sulfated when expressed by tumor cells other than NZB1.1 remains to be determined. However, based on the sulfation patterns of D^d expressed by the tumor cell lines A20.Cy and S49.1, it seems that only certain subsets of D^d expressed by these cells are likely to be sulfated.

We found the sulfo-radiolabeled D^d of NZB1.1 to be Endo H resistant, but PNGase F sensitive, indicating that D^d sulfation is a post-medial Golgi modification. These results are in agreement with previous studies indicating that sulfation of both carbohydrate and tyrosine occurs in the *trans*-Golgi network (Rosa et al., 1992). Thus, sulfation may be the last modification of D^d before it is expressed at the cell surface. The data presented in this study do not distinguish which carbohydrate residue(s) on D^d is sulfated. Sulfate modification can be quite complex, since it has been reported to occur on a variety of carbohydrate moieties, including penultimate or terminal galactose/N-acetylgalactosamine, as well as peripheral or core N-acetylglucosamine (GlcNAc) on *N*-linked carbohydrates (Elbein. 1987; Green et al., 1985; Hoogewerf and Bensadoun. 1991). It has been proposed that a hexose-6-SO_4 is part of the carbohydrate structure recognized by Ly-49A because soluble sulfated hexoses can inhibit this interaction (Daniels et al., 1994b). Since all major glycoforms of D^d expressed by NZB1.1 are sulfated, it can be speculated that the sulfate group is added to a carbohydrate moiety that is found in all of them. Thus, one possibility is the sulfate is added to one of the *N*-linked oligosaccharide core GlcNAc residues. It was observed that after treatment with 1-deoxymannojirimycin or swainsonine, inhibitors of mannosidase I and II, respectively (which interfere with oligosaccharide processing events required for conversion to complex oligosaccharide forms), D^d-expressing cells were still able to bind efficiently to Ly-49A-expressing cells (Daniels et al., 1994b). It has been demonstrated that swainsonine treatment of MDCK cell still allows sulfation of *N*-linked core GlcNAc (Merkle et al., 1985). This might explain why swainsonine and perhaps 1-deoxymannojirimycin have no effect on D^d interaction with Ly-49A, since they might still allow sulfation of an *N*-linked core GlcNAc. However, the exact carbohydrate unit(s) that is sulfated on D^d *N*-linked carbohydrate expressed by various cell lines remains to be determined.

Possible involvement of sulfated carbohydrate(s) on D^d and Ly-49A interaction. Oligosaccharide sulfation requires the sulfate donors PAPS and specific sulfoglycosyltransferases, and is sensitive to chlorate ions which inhibit PAPS formation catalyzed by ATP-sulfurylase (Baeurele and Huttner. 1986). We have shown here that D^d sulfation is susceptible to $NaClO_3$ inhibition and that NZB1.1 cultured in the presence of this inhibitor exhibited reduced D^d-dependent binding to isolated Ly-49A, especially when Ly-49A density is limiting. These results indicate that sulfated carbohydrate moieties on D^d can play a role in this interaction. Although the density of Ly-49 molecules on NK cells has not been determined, it is likely to be relatively low (Karlhofer et al., 1992; Daniels et al., 1994a; Brennan et al., 1994). Our solid phase adhesion assay employing isolated Ly-49A allow us to titrate Ly-49A through low densities that may be in the range expressed on NK cells (Chapter II; Chang et al., 1996). Since cell adhesion is most sensitive to $NaClO_3$ at a low density of Ly-49A, it is possible that sulfation of D^d may play a significant role in the interaction of D^d with Ly-49A molecules expressed on NK cells.

It is likely that the membrane-distal carbohydrate recognition domain of Ly-49A is involved in its interaction with the sulfated carbohydrate(s) of D^d; however, the precise role that sulfated oligosaccharide(s) on D^d play(s) in Ly-49A binding remains unclear. The sulfate carbohydrates may enhance the affinity, but perhaps not provide class I MHC allele specificity to the interaction. It is possible that class I MHC sulfated carbohydrate moieties strengthen the initial contact, and subsequent interaction with Ly-49A is determined by the class I MHC polypeptide backbone. The interaction may be analogous to class I MHC interaction with calnexin in the ER (Ware et al., 1995; York and Rock. 1996). Here, initial calnexin interaction appears to be dependent on an oligosaccharide intermediate expressed on class I MHC in the ER compartment, and subsequent stable contact is based on protein-protein interaction (Ware et al., 1995). Alternatively, and probably less likely, sulfated carbohydrates expressed on class I MHC may play a prominent role in allele specificity of Ly-49 recognition, possibly due to allele-specific oligosaccharide microheterogeneity (Swiedler et al., 1985).

Preliminary results from several laboratories suggest that individual Ly-49 family members recognize subsets of class I MHC alleles, and their specificities can partially overlap. For instance, D^d is shown to be a ligand for Ly-49A and Ly-49G2 (Kane. 1994; Daniels et al., 1994a; Mason et al., 1995). It will be of interest to determine whether sulfation affects class I MHC interaction with different Ly-49 members, e.g., Ly-49A and G2 to the same extent or uniquely, depending on the specificity Ly-49 receptor. In addition, Ly-49 family members may efficiently recognize only certain subsets of sulfated D^d. Although we have shown that D^d expressed by Yac-1 is sulfated, this cell line is sensitive to Ly-49A$^+$ NK cell lysis (Karlhofer et al., 1992), and is reported to be unable to stably bind Ly-49A-expressing cells (Brennan et al., 1994). Since Ly-49A interaction with D^d is peptide dependent but not peptide specific, bound peptide is not likely to account for these results. Instead, perhaps in addition to expressing relatively low levels of D^d, Yac-1 expresses sulfated D^d subsets or glycoforms that cannot stably interact with Ly-49A.

Given the complexity of *N*-linked carbohydrates on murine class I MHC molecules, the type and extent of sulfation may provide another level of regulation for Ly-49 members interaction with class I MHC molecules.

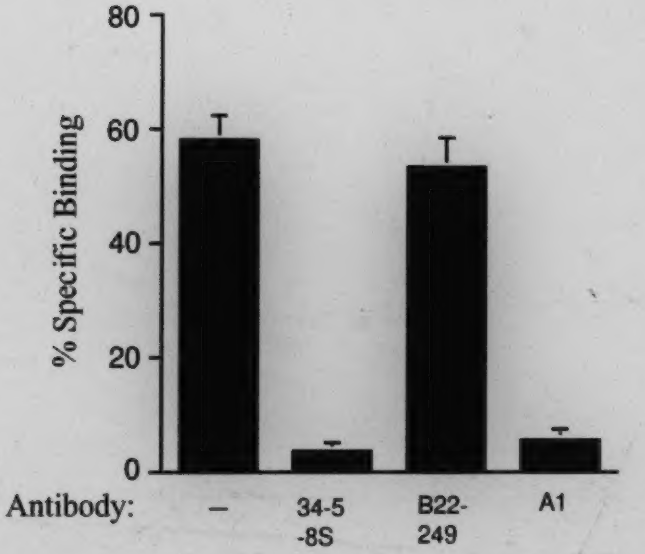


Figure 3-1. H-2D^d-expressing T lymphoma, NZB1.1, binds to plastic immobilized Ly-49A. The ^{51}Cr-labeled NZB1.1 cells were preincubated in medium for 20 min with 5 μg/ml of 34-5-8S or BB.249 mAb and then incubated for 1 h on microtitre wells immobilized with Ly-49A (10 ng). In the case of A1, the plate wells were preincubated with this mAb for 20 min before addition of untreated cells. Data are expressed as mean percent binding minus nonspecific cell adhesion to BSA-coated plate (<5%), with SD determined from triplicate wells.

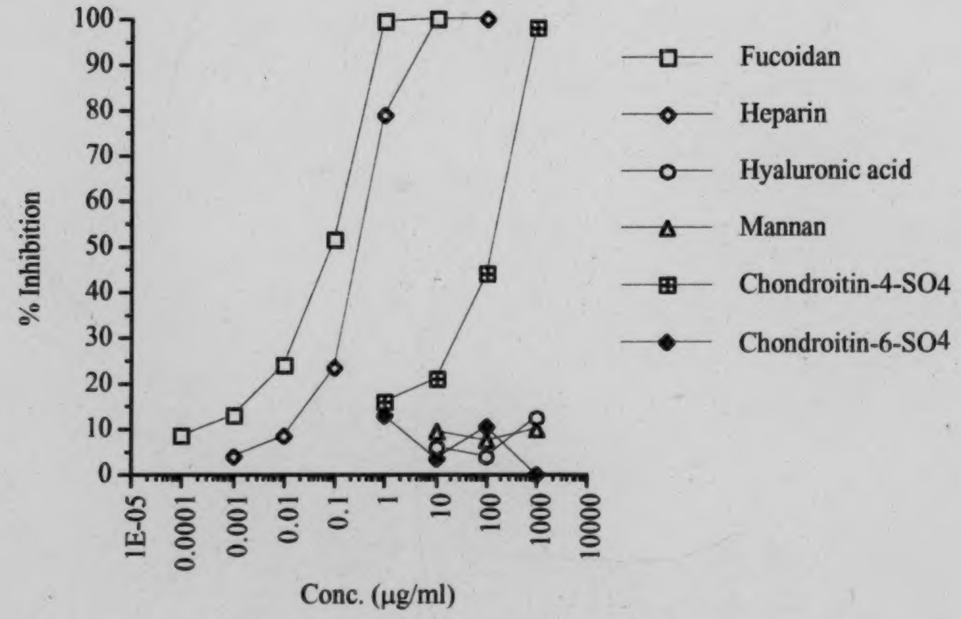


Figure 3-2. Binding of T lymohoma NZB1.1 to immobilized Ly-49A is inhibited by sulfated polysaccharides. ^{51}Cr-labeled NZB1.1 were added to microtitre wells coated with 10 ng of Ly-49A in the presence of the indicated polysaccharides. NZB1.1 binding to isolated Ly-49A in the absence of polysaccharide was 68%. Each polysaccharide concentration determine was carried out in duplicate. Nonspecific cell adhesion to BSA-caoated wells was less than 1%.

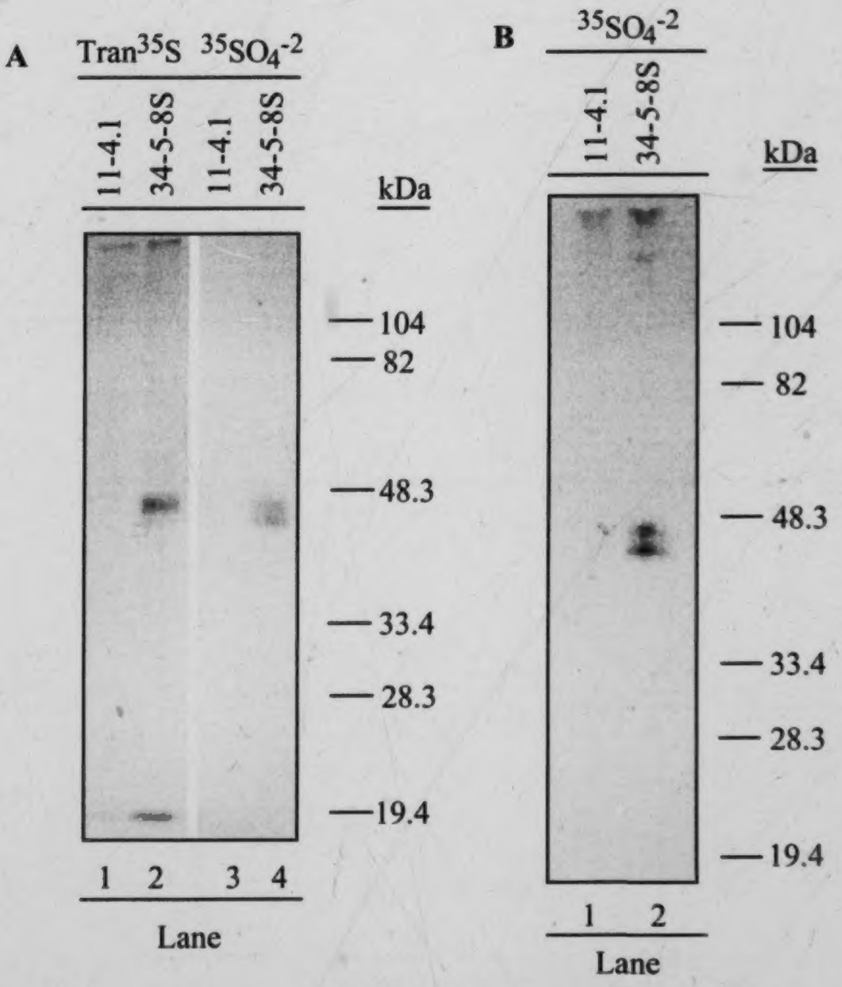

A
Tran35S
35SO4-2
11-4.1
34-5-8S
11-4.1
34-5-8S
kDa
104
82
48.3
33.4
28.3
19.4
1 2 3 4
Lane
B
35SO4-2
11-4.1
34-5-8S
kDa
104
82
48.3
33.4
28.3
19.4
1 2
Lane

Figure 3-3. NZB1.1 T lymphoma expresses sulfated forms of H-2D^d. The Tran^{35}S labeling of theT lymphoma cell line, NZB1.1, was conducted in Met-free RPMI for 8 h (A). Alternatively, Na$_2$^{35}SO$_4$ labeling was performed for 8 h in Fischer's medium (A), or sulfate-free DMEM (B), supplemented with 10% dialyzed FCS. H-2D^d was immunoprecipitated with 34-5-8S mAb or 11-4.1 as isotype control, and the the immunoprecipitated proteins were resolved by 10% SDS-PAGE gels. The gels were then treated with EN^{3}HANCE amplifier, dried and developed by autoradiography.

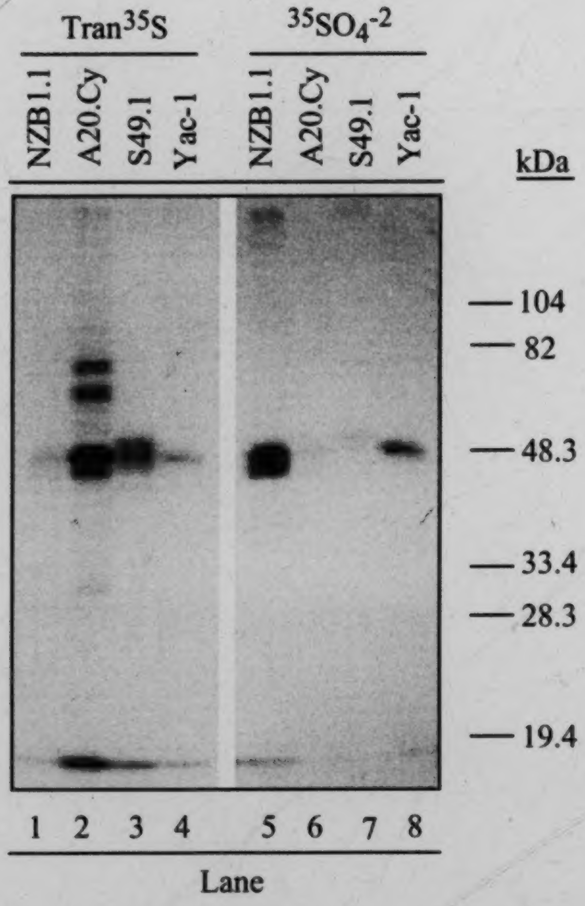

Tran35S
35SO4-2
NZB1.1
A20.Cy
S49.1
Yac-1
NZB1.1
A20.Cy
S49.1
Yac-1
kDa
104
82
48.3
33.4
28.3
19.4
1 2 3 4 5 6 7 8
Lane

Figure 3-4. Detection of sulfated H-2D^d expressed by a variety of tumor cell lines. Tran^{35}S and Na$_2$^{35}SO4 labeling was conducted in parallel for 8 h in Met-free RPMI and Fischer's medium, respectively. H-2D^d was immunoprecipitated using the D^d-specific mAb 34-5-8S, and then resolved on 10% SDS-PAGE gel. The gels were then treated with EN^{3}HANCE amplifier, dried and developed by autoradiography.

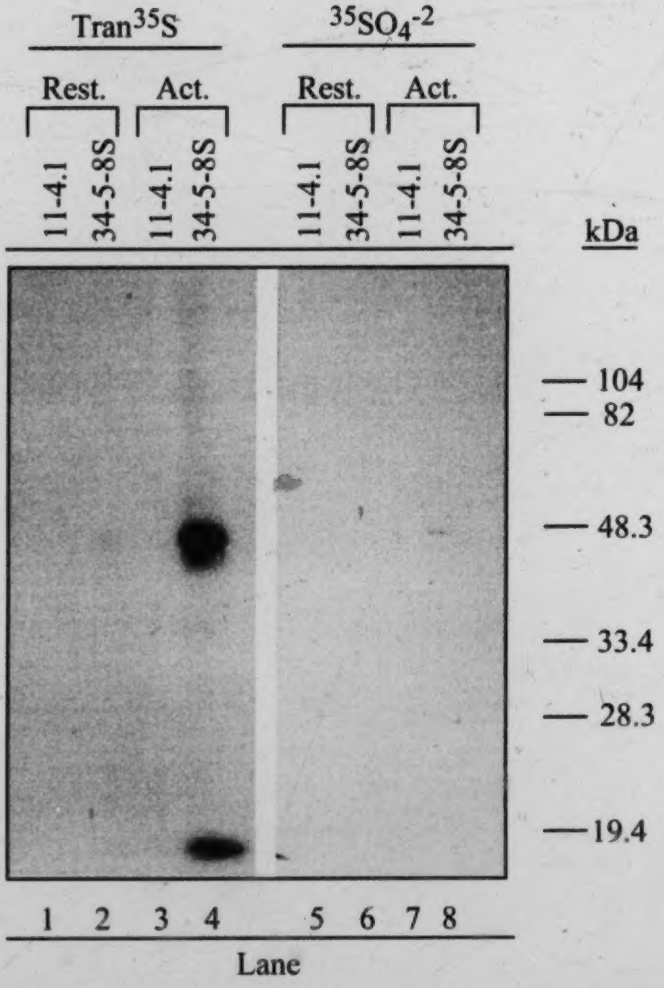

Tran35S
35SO4-2
Rest.
Act.
Rest.
Act.
11-4.1
34-5-8S
11-4.1
34-5-8S
11-4.1
34-5-8S
11-4.1
34-5-8S
kDa
104
82
48.3
33.4
28.3
19.4
1 2 3 4 5 6 7 8
Lane

Figure 3-5. Expression of sulfated H-2D^d by Con A-activated T lymphocytes from female Balb/c mice. Resting (Rest.) and Con A-activated (Act.) Balb/c T lymphocytes were incubated with Tran^{35}S and Na$_2$^{35}SO$_4$ in parallel for 8 h. The H-2D^d was immunoprecipitated by the 34-5-8S mAb from cell lysates and run on a 10% SDS-PAGE gel next to 11-4.1 isotype control immunoprecipitates. The gels were then treated with EN^{3}HANCE amplifier, dried and developed by autoradiography.

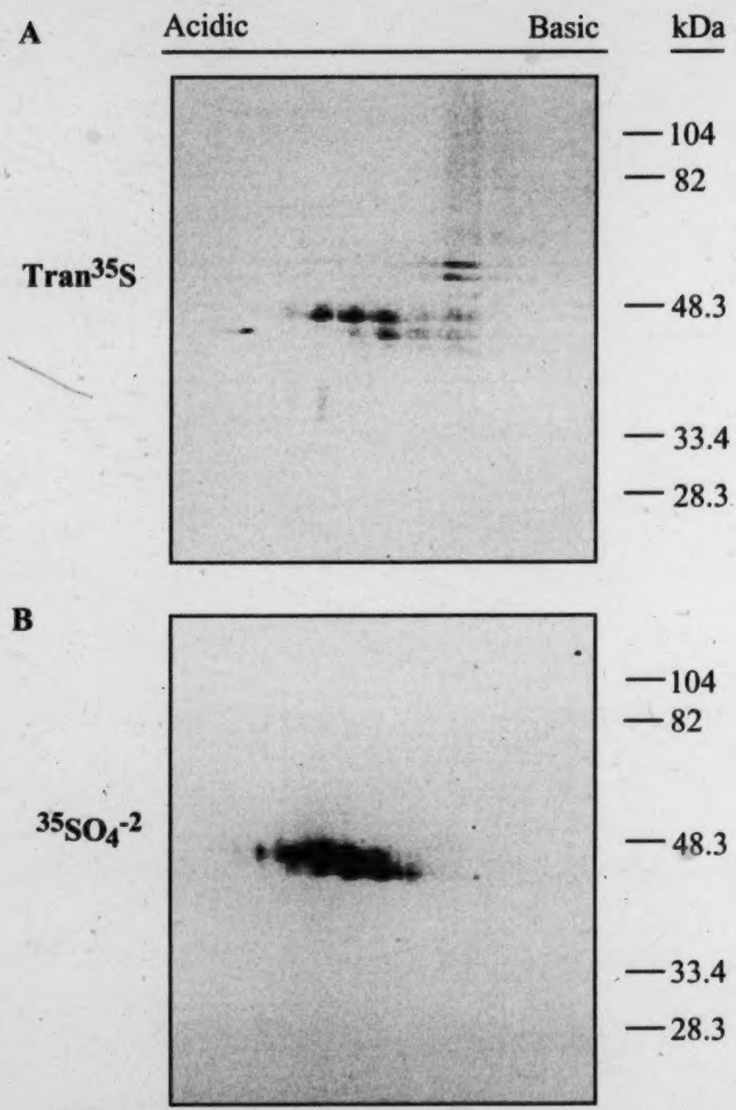

A
Acidic
Basic
kDa
Tran35S
104
82
48.3
33.4
28.3
B
35SO4-2
104
82
48.3
33.4
28.3

Figure 3-6. All H-2D^d glycoforms from NZB1.1 were sulfated as detected by two-dimensional PAGE analysis. The immunoprecipitated Tran^{35}S (A) and Na$_2{}^{35}$SO$_4$-labeled (B) H-2D^d from NZB1.1 were first resolved by IEF for 4800 V-h in tube gels. This was then followed by a second dimension separation by standard 10% SDS-PAGE gel. The gels were treated with EN^{3}HANCE amplifier, dried and visualized by autoradiography. The pI range shown on the autoradiaogram is from 5.3 to 6.5 (*left to right*).

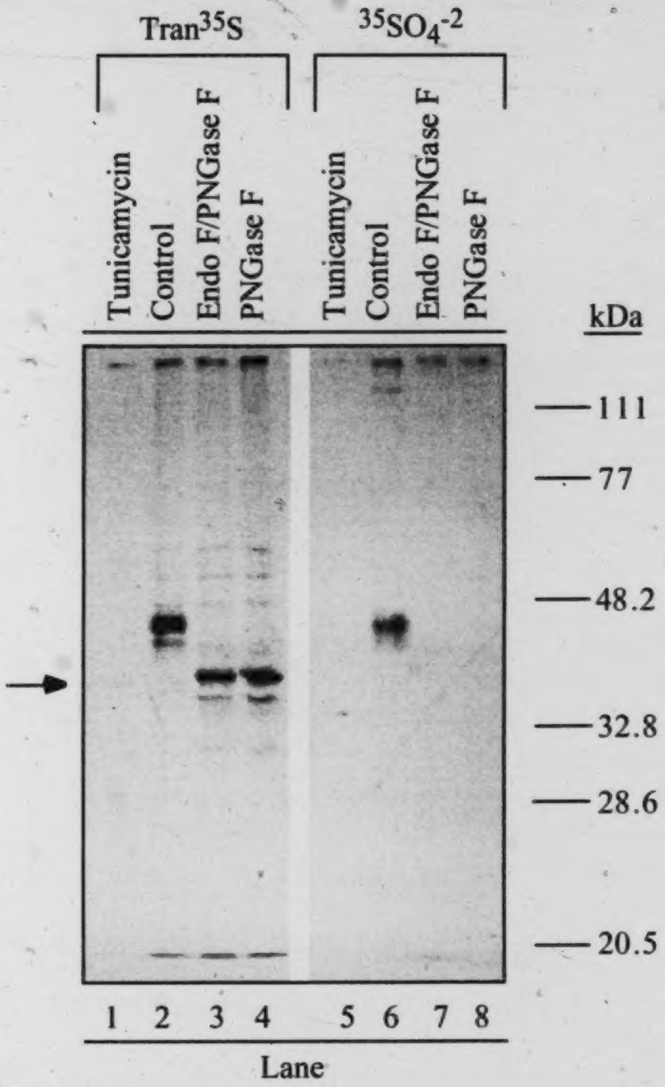

Tran^{35}S
^{35}SO$_4$$^{-2}$
Tunicamycin
Control
Endo F/PNGase F
PNGase F
Tunicamycin
Control
Endo F/PNGase F
PNGase F
kDa
111
77
48.2
32.8
28.6
20.5
1 2 3 4 5 6 7 8
Lane

Figure 3-7. Sulfation of *N*-linked oligosaccharide(s) on H-2D^d isolated from from NZB1.1. NZB1.1 were incubated with or without 1 µg/ml of the *N*-linked glycosylation inhibitor tunicamycin for 7 h in the presence of Na$_2$^{35}SO$_4$ or Tran^{35}S label. The labeled H-2D^d were then immunoprecipitated with D^d-specific mAb 34-5-8S. For enzymatic removal of *N*-linked carbohydrates, the Tran^{35}S or Na$_2$^{35}SO$_4$-labeled D^d obtained from untreated NZB1.1 lysates were first alkylated before treated with or without (control) 0.3 mU of Endo F/PNGase F or 3 U PNGase F for 20 h at 37°C as described in *Materials and Methods*.

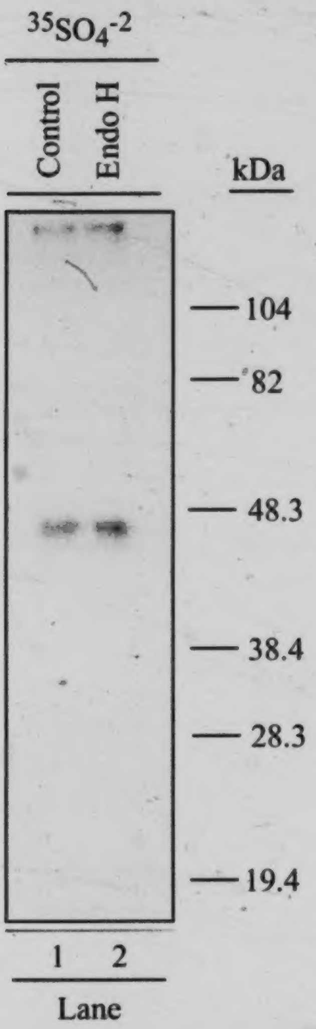
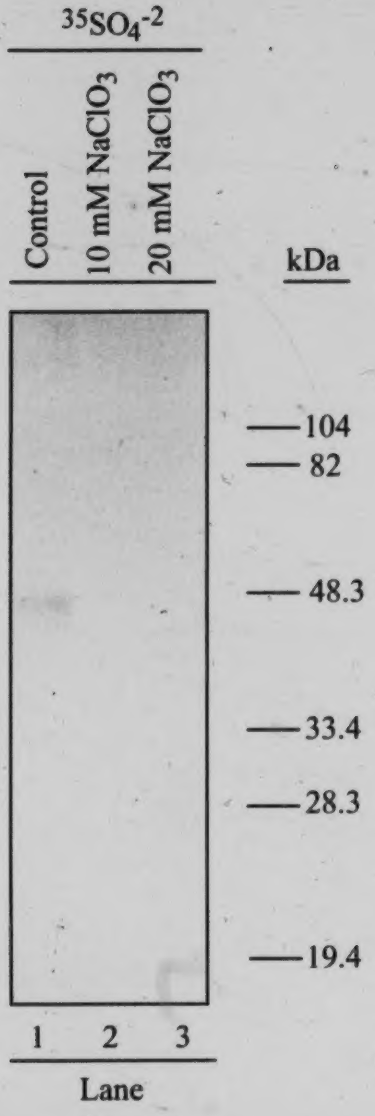

$^{35}SO_4^{-2}$
Control
Endo H
kDa
104
82
48.3
38.4
28.3
19.4
1
2
Lane

Figure 3-8. H-2D^d sulfation occurs on mature *N*-linked oligosaccharide(s). $Na_2{}^{35}SO_4$-labeled D^d immunoprecipitated from NZB1.1 was treated with or without (control) 3 mU of EndoH, which removes immature *N*-linked carbohydrates, for 20 h at 37°C. Immunoprecipitated materials were resolved on 10% SDS-PAGE gels. Radiolabeled H-2D^d were detected by autoradiography.



$^{35}SO_4^{-2}$
Control
10 mM $NaClO_3$
20 mM $NaClO_3$
kDa
104
82
48.3
33.4
28.3
19.4
1
2
3
Lane

Figure 3-9. Inhibition of NZB1.1 H-2D^d sulfation by $NaClO_3$. Immunoprecipitation of D^d from NZB1.1 cell lysates was performed following an 8 h labeling with $Na_2{}^{35}SO_4$ without addition of $NaClO_3$ (*lane 1*), or with 10 mM or 20 mM $NaClO_3$ in Fischer's medium (*lanes 2 and 3*, respectively). The immunoprecipitated proteins were then resolved on 10% SDS-PAGE, and the gel was then treated with EN^{3}HANCE, dried and developed by autoradiography.

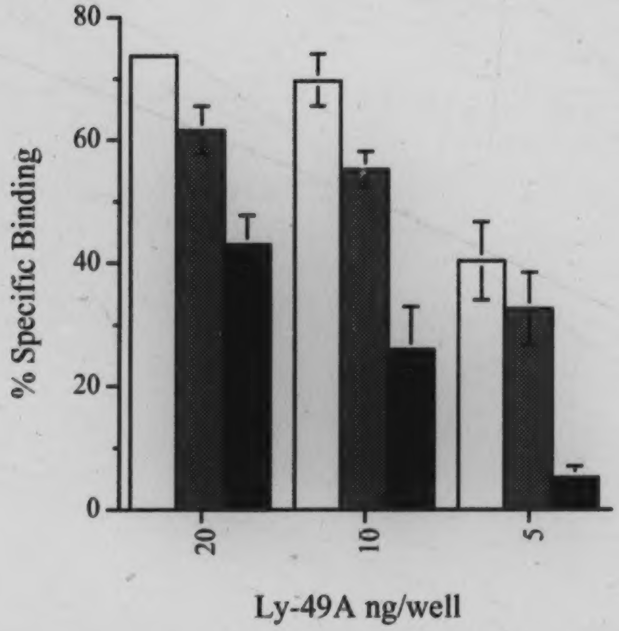


Figure 3-10. Inhibition of H-2D^d-dependent NZB1.1 binding to immobilized Ly-49A by NaClO$_3$. NZB1.1 cells were grown in sulfate-free DMEM supplemented with: 1). 0.8 mM MgSO$_4$ (open bar), 2). 0.8 mM MgCl$_2$ (gray bars), or 3). 0.8 mM MgCl$_2$ and 20 mM NaClO$_3$ (black bars). NZB1.1 binding to immobilized Ly-49A at the indicated densities was determined after 1 h of incubation. Results are expressed as mean percent binding minus nonspecific cell adhesion to BSA-coated wells (<5%), with SD determined from triplicate wells.

Table 3-1

Expression of H-2D^d on NZB1.1 cultured in Fischer's medium and DMEM and after exposure to various concentrations of NaClO$_3$

NZB1.1 Treatment	MFI (in Fischer's medium)	MFI (in DMEM)
Untreated control	22.88	22.88
5 mM NaClO$_3$	22.67	23.29
10 mM NaClO$_3$	22.47	23.29
20 mM NaClO$_3$	22.88	22.88

In all instances, AF-6.88.5, anti-K^b, was used as an isotype control, whereas the MFI of H-2D^d expression was determined by 34-5-8S. The MFI of the isotype control for NZB1.1 grown in Fischer's medium and DMEM were 3.13 and 2.97, respectively.

CHAPTER IV

GENERATION AND CHARACTERIZATION OF TWO NEW MONOCLONAL ANTIBODIES SPECIFIC FOR Ly-49G2^{B6}

(Part of the data presented in this chapter is submitted for publication).

A. Introduction

With nine already known Ly-49 family members, and possibly a few more remaining to be described (McQueen. et al., 1998; Kane et al., unpublished observation) the studies of NK cell regulatory mechanisms through these receptors has become tremendously complicated. To date only a handful of them have been studied and characterized in any detail. Among these Ly-49 NK cell receptors, only Ly-49A, C, D and G2 have been clearly demonstrated to regulate NK cell cytotoxic activity. Furthermore, only Ly-49A and C have been shown to physically interact with class I MHC molecules (Kane. 1994; Daniels et al., 1994a; Takei et al., 1997). The lack of Ly-49-specific mAbs is a major factor that hinders the study of these NK cell regulatory receptor functions. This problem is further compounded by the fact that most of the available mAbs exhibit some form of cross reactivity. For example, the mAb SW5E6 recognizes both Ly-49C and I of B6 and Balb/c origin (Takei et al., 1997), and 12A8 recognizes Ly-49A and D (Mason et al., 1996). Similarly, 4D11 was first thought to be specific for Ly-49G2, but is now shown to cross react with Ly-49A of B6 and Balb/c origin (Salcedo et al., 1997; Takei et al., 1997). In addition, the newly available mAb, K2D, is also shown to react with Ly-49G2 and another Ly-49-like molecule(s) (Kubota. 1997). With the likely existence of additional Ly-49 NK cell receptors, it is possible that the available mAbs can also cross react with these "uncharacterized" Ly-49 molecules. Consequently, the study of the ligand specificity and the regulatory functions of these Ly-49 receptors has become rather complicated.

The Ly-49G2 receptor has been implicated to be an H-2D^d and L^d-specific inhibitory receptor (Mason et al., 1995). In addition, it is further shown that Ly-49G2 also recognizes D^r and an undescribed H-2^k product(s) (Thaddeus et al., 1999). However, a cell-cell adhesion study has yielded seemingly conflicting results, which showed no detectable binding of H-2^{b,d,k,s}-expressing tumor cell lines to COS-1 transiently expressing Ly-49G2 (Takei et al, 1997). In addition, the view of L^d as a ligand for Ly-49G2 is being challenged by the recently published data, as L^d expression failed to protect target cells from lysis by Ly-49G2$^+$ NK cells or down regulate Ly-49G2 expression (Johansson et al., 1998). Since studies of Ly-49G2 ligand specificity were all carried out using 4D11, it is not unreasonable to assume that these conflicting data were due to the use of this cross reactive mAb. Theoretically, one can argue that the reported inhibitory effect of D^d and L^d on Ly-49G2$^+$ NK cells sorted by using 4D11 could be caused by the contaminating Ly-49A$^+$ population. Furthermore, an early interesting observation regarding the reactivity of 4D11 is that it was originally reported to recognize a triggering molecule on NK cells (Mason et al., 1994). It is entirely possible that besides Ly-49A and G2, 4D11 also recognizes some undescribed activating Ly-49 receptors. Therefore, sorting NK cells based on 4D11 reactivity could potentially isolate NK cell subsets expressing D^d and L^d inhibitory receptor(s) which are preferentially co-expressed on these 4D11$^+$ NK cells. As a result, D^d, D^r, L^d and the reported H-2^k product(s) might not exert their inhibitory effect directly through Ly-49G2.

To facilitate the study of NK cell functions, we have attempted to generate mAbs against murine NK cell specific markers. By immunizing Balb/c mice with B6 A-LAK and fusing the splenocytes with NS-1 and Sp2/0 myelomas, we isolated two hybridomas, Cwy-3 and Ck1, which both secrete IgG$_1$ directed against Ly-49G2. Therefore, with these two new mAbs, the ligand specificities and the regulatory functions of Ly-49G2 can be properly evaluated.

B. Materials and Methods

Mice. Female AKR/J (H-2^k), CBA/J (H-2^k), NZB/BinJ (H-2^d), 129/J (H-2^b) and B6x129/J F_1 (H-$2^{b/b}$) mice were purchased from the Jackson laboratory (Bar Harbor, ME). Female C57BL/6 (B6, H-2^b) mice were purchased from both the Jackson laboratory and the Charles River laboratories (Wilmington, MA). Both female and male B6 Rag-$1^{-/-}$ and female Balb/c (H-2^d) mice were obtained from the University of Alberta animals breeding facility (Edmonton, AB., Canada). All mice used were eight to twelve-week of age, except NZB/BinJ, which were nine months of age.

mAbs. BB7.1 (mouse IgG_1), anti-human HLA-B27, 4D11 (rat IgG_{2a}), anti Ly-49A and G2, and Y13-238 (rat IgG_{2a}), anti-rat ras were obtained from ATCC (Manassas, VA). A1 (mouse IgG_{2a}), anti-Ly-49A, was provided by Dr. J. Allison (University of California Berkeley, Berkeley, CA). The Cwy-3 (IgG_1) and Ck1 (IgG_1) mAbs were generated in our laboratory. All purified mAbs were obtained from hybridoma supernatants by ammonium sulfate precipitation and dialyzed against PBS. SW5E6 (mouse IgG_{2a}), anti-Ly-49C/I was purchased from PharMingen (San Diego, CA). The FITC-conjugated goat anti-rat IgG and FITC-conjugated rat anti-mouse IgG were purchased from Jackson ImmunoResearch Laboratory (West Grove, PA).

Cell lines. ANA-1, a macrophage cell line derived from C57BL/6, was a gift from Dr. M. Belosevic (University of Alberta, Edmonton, AB., Canada). CTLL-2, EL-4, NS-1 and Sp2/0 were kindly provided by Dr. H. Ostergaard (University of Alberta, Edmonton, AB., Canada). RMA was a gift from Dr. W. Jefferies (University of British Columbia, Vancouver, B.C., Canada). C1498, IC-21, TIMI.4 and WEHI-164 were obtained from ATCC (Manassas, VA). ANA-1, EL-4, NS-1 and Sp2/0 were cultured in DMEM, supplemented with 5% heat-inactivated BCS, 20 mM Hepes, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. CTLL-2, C1498, IC-21, RMA and WEHI-164 were propagated in RPMI with the same supplements as in DMEM. For CTLL-2, 20 units/ml of rIL-2 was also added to the culture.

Generation of adherent lymphokine-activated killers (A-LAKs). A-LAKs from various mouse strains were generated as previously described (Chang et al., 1999). Briefly, RBC-depleted splenocytes were passaged through nylon wool columns to obtain NWNA cells. These unbound cells were then cultured in complete RPMI/10% FCS with 800 units/ml of rIL-2. For those A-LAK derived from B6 Rag-1$^{-/-}$ and B6x129/J F$_1$ Rag-1$^{-/-}$ mice, a similar protocol was used, except the RBC-depleted splenocytes were not passed through a nylon wool column.

Immunization and fusion. Female Balb/c mice were immunized seven to nine times with B6 A-LAK in the course of five to nine months, with 2x10^7 A-LAKs for each injection. The route of immunization was i.p., except the last two injections which were administered through i.v. injection. Three days after the last injection, spleen cells were removed and depleted of RBCs. The spleen cells were then fused with NS-1 or Sp2/0 myelomas as described (Galfre et al., 1977). Briefly, a 10:1 fusion ratio of spleen cells to NS-1 or Sp2/0 was fused with 50% polyethylene glycol solution. Upon removal of the fusogen, the cell mixture was resuspended in DMEM supplemented with 20% FCS, 1x OPI (oxaloacetate, sodium pyruvate and insulin) and 1x HAT (100 μM hypoxanthine, 0.4 μM aminopterin and 16 μM thymidine), and 200 μl of the cell suspension was added to each well of a 96-well microtitre plate. After 7 to 10 days of plating, wells were examined for cell growth. Supernatants from these hybridomas were tested for the presence of IgG by sandwich ELISA. For those containing IgG, their abilities to stain B6 A-LAKs were further analyzed by FACS analysis.

Immunofluorescence staining. A half to one million A-LAKs or tumor cells were stained with 2.5 - 5 μg of purified mAbs or 100 - 200 μl of hybridoma supernatants for 30 min at 4°C. After extensive washing, the cells were counter stained with 200 μl of 1:100 dilution of FITC-conjugated rat anti-mouse IgG or FITC-conjugated goat anti-rat IgG for another 30 min at 4°C. The samples were further washed and fixed with 2% paraformaldehyde before analysis by FACScan. In all instances, except when the primary mAbs were rat in origin or for COS-7 transfectants immunofluorescence staining, the cells were pre-incubated with 10 μg/ml of 2.4G2 for 30 min at 4°C and washed before staining with the primary mAbs.

Immunoprecipitation and SDS-PAGE. B6 A-LAKs were cell surface biotinylated as described previously (Boxberg et al., 1990). Ten million of the biotinylated cells were lysed in 1 ml of lysis buffer (PBS containing 1% NP-40, 1 mM PMSF and 5 mM iodoacetamide, pH 7.2) at 4°C. The lysate was pre-cleared twice with 30 μl of protein G packed beads and 5μl of normal Balb/c serum prior to immunoprecipitation with 10 μg of Cwy-3 or BB7.1 and protein G-conjugated beads for 2 h. The beads were then washed five times with lysis buffer containing 1% DOC. The immunoprecipitated proteins were eluted by incubating beads with 50 μl of TRIS-glycine buffer, pH 2.9, at 100°C for 4 min, and then neutralized with a pre-determined volume of Tris buffer, pH 8.9. The immunoprecipitated samples were resolved on a 10% SDS-PAGE under both nonreducing and reducing conditions (Laemmli. 1970), and transferred to Immobilon-P. The immobilon-P membrane was then blocked with 4% BSA, and incubated with 1:20000 dilution of strepavidin-HRP. The proteins were then visualized by using enhanced chemiluminescence (ECL).

Transfection of COS-7. pC1-neo vector containing cDNA encoding the B6 alleles of Ly-49A through I were transfected into COS-7 by using Lipofectamine methods according to the manufacturer's (Gibco) recommendations. After 3 days of transfection, cells were harvested, stained with Cwy-3 and analyzed by FACScan.

Separation of B6 A-LAKs into Cwy-3$^+$ and Cwy-3$^-$ populations by magnetic beads. After 5 to 6 days of culture, A-LAKs were harvested and incubated with 40 μg/ml of Cwy-3 at 4°C for 30 min. Excess mAbs were then removed by extensive washing. Rat anti-mouse IgG-conjugated Dynabeads were then added at the concentration of 4 beads per cell to the Cwy-3-treated A-LAKs. The bead and cell mixture was then further incubated for another half an hour at 4°C. The B6 A-LAKs were eventually separated by magnet attraction into Cwy-3$^+$ and Cwy-3$^-$ populations, and recultured in the presence of 800 units/ml of rIL-2 for another 3 to 4 days before stained with various mAbs and analyzed by FACScan.

Isolation of EL-4/Cwy-3$^+$ by magnetic beads. EL-4 cells were first incubated with 40 μg/ml of Cwy-3 for 30 min at 4°C. After extensive washing, the cells were further incubated with sheep anti-mouse IgG-conjugated Dynabeads, at the concentration of 4 beads per cell, for another 30 min at 4°C. The Cwy-3$^+$ EL-4 cells were then positively selected by magnet attraction. After resting in culture for 48 hrs, the same procedures were repeated to further expand the EL-4/Cwy-3$^+$ population. The selection process was then repeated until high levels of Ly-49G2 expression were detected by Cwy-3 staining.

^{51}Cr release assay. The ^{51}Cr-labeled WEHI-164 was incubated with the Cwy-3$^+$ and Cwy-3$^-$ B6 A-LAKs at various E:T ratios in triplicate with the presence of 20 μg/ml of the indicated mAbs for 4 h at 37°C. The percentage specific lysis was determined as [(experimental release - spontaneous release)/(maximum release - spontaneous release)] x 100.

C. Results

Screening for mAbs that recognize cell surface molecules on B6 A-LAKs. Spleen cells from two female Balb/c mice immunized with B6 A-LAKs were fused with either murine myelomas NS-1 or Sp2/0 in two independent fusions. The supernatants from the IgG producing hybridomas were tested for their abilities to stain B6 A-LAK by FACS analysis. Two hybridomas, Cwy-3 ($IgG_{1,\ \kappa}$) and Ck1 ($IgG_{1,\ \kappa}$), with NS-1 and Sp2/0 as fusion partners respectively, were found to stain a large population of B6 A-LAKs. To ensure clonality, the hybridomas were further subcloned by limiting dilution. FACS data showed that both Cwy-3 and Ck1 recognized approximately fifty percent of the B6 A-LAK (Fig. 4-1a, upper and lower panels). A similar staining pattern was also seen on A-LAK derived from B6 mice with Rag-$1^{-/-}$ phenotypes (Fig. 4-1b). Since Rag-$1^{-/-}$ mice do not support B and T cell maturation (Mombaerts et al., 1992), and with the same proportion of B6 Rag-$1^{-/-}$ A-LAKs stained positive as normal B6 A-LAKs, this strongly indicated that both of these mAbs could be recognizing markers found on a large subset of NK cells.

Correlation of Cwy-3 and Ck1 staining patterns with that of 4D11. To further examine the relation between the Cwy-3 and Ck1 defined cell surface markers, we sorted B6 A-LAK into Cwy-3^{+} and Cwy-3^{-} populations. After three to four days of culture, the cells were re-analyzed for their expression of the Cwy-3 and Ck1 defined markers. Cwy-3 staining indicated that the expression of the Cwy-3-specific marker was relatively stable after separation and short term reculture (Fig. 4-2). Surprisingly, Ck1 seemed to recognize the same subset of B6 A-LAKs as those defined by Cwy-3 (Fig. 4-2). For example, Ck1 only stained the Cwy-3^{+} but not the Cwy-3^{-} population. Therefore, these data seem to suggest that both of these mAbs might recognize the same cell surface marker on B6 A-LAK. When these Cwy-3^+ and Cwy-3^- A-LAKs were analyzed for their expression of Ly-49A by A1, it was found that approximately 20 to 30 % of both A-LAK populations expressed Ly-49A (Fig. 4-3, upper panel). Thus, this suggests that Cwy-3 is not specific for this particular NK cell receptor. However, when Cwy-3^+ and Cwy-3^- A-LAKs were stained by 4D11, a correlation with Cwy-3 and Ck1 staining patterns was revealed (Fig. 4-3, lower panel). For instance, the Cwy-3^+ A-LAK was highly positive for 4D11 staining, but only weakly on approximately 20% of the Cwy-3^- population (Fig. 4-3, upper panel). Since 4D11 is specific for both Ly-49A and G2, this residual staining on the Cwy-3^- population is probably due to the expression of Ly-49A, as indicated by the staining of A1 on both Cwy-3^+ and Cwy-3^- A-LAKs (Fig. 4-3, upper panel). Therefore, with the staining of 4D11 on Cwy-3^+ A-LAK and the presence of Ly-49A on both Cwy-3^+ and Cwy-3^- populations, the FACS data strongly imply that both Cwy-3 and Ck1 could be specific for Ly-49G2.

To further analyze the reactivity of both Cwy-3 and Ck1, the abilities of these mAbs to stain A-LAKs derived from other mouse strains were examined. The Cwy-3 mAb failed to stain the A-LAKs derived from AKR/J (H-2^k), CBA/J (H-2^k) and NZB/BinJ (H-2^d) (Fig. 4-4). However, approximately fifty percent of the 129/J (H-2^b) A-LAKs did stain positively with Cwy-3 (Fig. 4-4). For Ck1, besides B6 mice, the only other mouse strain tested was AKR/J. Similar to Cwy-3, Ck1 also failed to stain the A-LAK derived from this mouse strain (Table 4-1). Based on these staining patterns, both Cwy-3 and Ck1 do not seem to share the broad mouse strain distribution as seen in 4D11 staining (Table 4-1). Therefore, if these mAbs are indeed recognizing the same cell surface marker, it is likely that both Cwy-3 and Ck1 are recognizing a different epitope(s) than that seen by 4D11.

Reduction in expression of the Cwy-3 and Ck1-specific cell surface marker(s) with the introduction of the 129/J genetic background. With the limited mouse strains tested, the Cwy-3 defined epitope seems to be only found in H-2^b mice, B6 and 129/J (Fig. 4-1 and 4-4). Although these mouse strains yielded the same proportion of Cwy-3^+ A-LAKs, it is important to note that the B6 A-LAKs showed a higher MFI than 129/J A-LAKs (Fig. 4-4). In general, the B6 Cwy-3^+ A-LAK population shows a MFI of 200, whereas, 129/J Cwy-3^+ A-LAK only exhibits a MFI of 40. The reason for this reduction in MFI in 129/J A-LAKs remains to be further investigated. Since both B6 and 129/J mouse strains stained positive for Cwy-3, and Ck1 seemed to recognize the same subset of NK cells as those defined by Cwy-3, we therefore analyzed the expression of Cwy-3 and Ck1 defined marker(s) in B6x129/J F_1 Rag-$1^{-/-}$ mice. FACS data indicated that only approximately forty percent of the F_1 mice A-LAKs were stained positively by Cwy-3 and Ck1 (Fig. 4-5). Compared to the B6 Rag-$1^{-/-}$ A-LAKs (Fig. 4-1b), a reduction in both the percentage of Cwy-3^+ and $Ck1^+$ and MFI are also observed in the B6x129/J F_1 A-LAKs. These data suggested that with the introduction of the 129/J genetic background, expression of the Cwy-3 and Ck1-specific marker(s) could potentially be down regulated.

Cwy-3 is specific for B6 Ly-49G2. The staining pattern of Cwy-3 showed that it is a cell surface marker expressed by a subset of B6 and 129/J A-LAKs. To further characterize the specificity of this mAb, we immunoprecipitated the Cwy-3-reactive material from cell surface biotinylated B6 A-LAK cell lysates. Under both nonreducing and reducing conditions, no proteins were immunoprecipitated by the isotype control mAb, BB7.1 (Fig. 4-6, lanes 1 and 3). In contrast, Cwy-3 immunoprecipitated a protein with a M_r of approximately 86 kDa (Fig. 4-6, lane 2). Under reducing conditions, the protein showed a M_r of approximately 43 kDa (Fig.4-6, lane 4). However, even under reducing conditions, not all of the protein was fully reduced, as indicated by the presence of the remaining 86 kDa band (Fig. 4-6, lane 4). These immunoprecipitation data showed that Cwy-3 recognizes a disulfide-linked dimer expressed on a subset of B6 A-LAK. With the correlation in Cwy-3 staining with 4D11 using B6 A-LAK, this strongly suggested that Cwy-3 is specific for Ly-49G2.

To confirm this possibility, we transfected COS-7 with the expression vector pC1-neo containing the cDNA encoding the B6 allele of Ly-49 members. FACS analysis of the transfectants showed that Cwy-3 only stained COS-7 cells transfected with Ly-49G2^{B6} but not those transfected with other Ly-49 members such as Ly-49A, Ly-49C - I (Fig. 4-7). Although the staining of Ly-49G2^{B6} transfected COS-7 by Cwy-3 was relatively weak, similar percentage of Ly-49G2^{B6} expressing COS-7 was detected by 4D11 staining (data not shown). In addition, expression of Ly-49A and Ly-49C/I on COS-7 transfectants were also confirmed by A1 and SW5E6 staining (data not shown). Therefore, unlike 4D11 and K2D, Cwy-3 is specific for Ly-49G2 and exhibits no cross reactivity to other known Ly-49 family members.

Expression of Ly-49G2 on tumor cell lines and isolation of an EL-4 subline expressing Ly-49G2. It is known that some tumor cell lines such as EL-4 can express NK cell inhibitory receptors such as Ly-49A at high levels (Nagasawa et al., 1987). In addition, based on 4D11 staining, this tumor cell line has also been reported to express Ly-49G2 and used as a model to study the function of this inhibitory receptor (Ortaldo et al., 1998). If this is the case, this cell line might provide a constant source of Ly-49G2 that can facilitate the study of this NK cell inhibitory receptor. With the new anti-Ly-49G2-specific mAb, we therefore examined EL-4 and screened a panel of B6 derived tumors for their Ly-49G2 expression by using Cwy-3. Among the tumor cell lines analyzed, including ANA-1, CTLL-2, C1498, EL-4, IC-21, RMA and TIMI.4, only EL-4 was found stained positive by Cwy-3 (Fig. 4-8 and data not shown). However, only less than 5% of the EL-4 cells were weakly positive for Cwy-3 staining, but they were highly positive for both A1 and 4D11 staining (Fig. 4-8). This is in sharp contrast with those previously reported results, in which it was shown that EL-4 expresses high levels of Ly-

49G2 (Ortaldo et al., 1998). Since the conclusion was based on 4D11 staining, it is likely the mAb simply detecting Ly-49A on EL-4 instead of Ly-49G2. Thus, these results further demonstrated the cross reactivity of 4D11 and caution has to be exercised when interpreting data regarding Ly-49G2 functions by using EL-4 as a model.

Since Cwy-3 seems to stain an extremely small population of EL-4, we attempted to isolate an EL-4 subline that expresses substantial levels of Ly-49G2 by using Dynabeads. After multiple rounds of magnetic selection by Cwy-3, EL-4 expressing intermediate levels of Ly-49G2, designated as EL-4/Cwy-3$^+$, were obtained (Fig. 4-9). With this newly selected EL-4 subline, we further examined the relationship between Cwy-3 and Ck1. The staining pattern of Ck1 on EL-4/Cwy-3$^+$ correlated with that of Cwy-3 (Fig.4-9), further supporting the notion that Ck1 also recognizes Ly-49G2. It is important to note that the expression of Ly-49G2 by EL-4/Cwy-3$^+$ shown in Fig. 4-9 was the highest that we have seen on this cell line. It seems that this T lymphoma does not support a high level of Ly-49G2 expression as it does for Ly-49A, for there were times where EL-4/Cwy-3$^+$ has shown a much lower staining by Cwy-3. Furthermore, during each step of the selection process, there was always a large population of EL-4 that failed to be bound by the magnetic beads. Therefore, it is possible that intrinsically, EL-4 simply does not support high level expression of Ly-49G2

Cytotoxic functions of the Ly-49G2$^+$ population selected by Cwy-3. These new Ly-49G2-specific mAbs provided us an opportunity to re-examine the protective effect of D^d on Ly-49G2$^+$ NK cells. The ability of sorted Cwy-3$^+$ and Cwy-3$^-$ B6 Rag-1$^{-/-}$ A-LAKs to lyse the Fc receptor negative target cell, WEHI-164, was determined by *in vitro* ^{51}Cr release assay. The data showed that WEHI-164 was rather resistant to lysis by both Cwy-3$^+$ and Cwy-3$^-$ A-LAKs (Fig. 4-10). However, in the presence of Cwy-3, Ck1 or 4D11, there was an increase in lysis of WEHI-164 by Cwy-3$^+$ but not Cwy-3$^-$ populations. Since WEHI-164 does not express any Fc receptors, this low level of restoration in lysis is not mediated by ADCC. Furthermore, the Ly-49A expressed on these populations also does not contribute to this inhibition of lysis, since addition of A1 failed to induce lysis of WEHI-164 (Fig. 4-10).

D. Discussion

In an attempt to generate mAbs against B6 A-LAK cell surface molecules, we have isolated two hybridomas, Cwy-3 and Ck1, which secrete IgG specific for B6 NK cell surface markers. COS-7 transfection data indicated that Cwy-3 specifically recognizes Ly-49G2^{B6}, but not the other Ly-49 receptors. As for Ck1, the data regarding its specificity is only circumstantial. Based on its staining pattern, it correlates with that of Cwy-3 and 4D11. It is only logical to conclude that Ck1 is also specific for Ly-49G2. This conclusion is further supported by preliminary ELISA data which showed that both Cwy-3 and Ck1 react with immunoaffinity purified Ly-49G2 (data not shown). However, whether Ck1 exhibits cross reactivity to other Ly-49 receptors remains to be determined.

FACS analysis showed that both of these mAbs stained approximately fifty percent of the B6 and B6 Rag-1$^{-/-}$ A-LAKs (Fig. 4-1A and B). In addition to B6 derived A-LAKs, Cwy-3 also stained A-LAKs generated from 129/J (H-2^{b}) and B6x129/J F$_1$ Rag-1$^{-/-}$ (H-2$^{b/b}$) mice, but not those derived from mouse strains with other H-2 haplotypes (Fig. 4-4, 4-5 and Table 4-1). As for Ck1, only limited mouse strains were examined. Nevertheless, it also stained B6x129/J F$_1$ Rag-1$^{-/-}$ but not AKR/J derived A-LAKs (Fig. 4-5 and Table 4-1). Since both Cwy-3 and Ck1 were generated by immunizing Balb/c (H-2^{d}) mice with B6 A-LAKs, they were expected not to recognize Ly-49G2$^{Balb/c}$. Therefore, both Cwy-3 and Ck1 showed different properties from the two known anti-Ly-49G2 mAbs, 4D11 (Table I) and K2D.

Besides Ly-49G2, both 4D11 and K2D are known to react with other Ly-49 molecules. For instance, the cross reactivity of 4D11 is not only seen on transfectants as previously reported (Salcedo et al., 1997; Takei et al., 1997), but also can be detected on

A-LAKs and on tumor cells (Fig. 4-3 and 4-8). As for K2D, in addition to Ly-49G2, it also recognizes other Ly-49-like molecule(s) (Kubota. 1997). Since they stain A-LAKs derived from other mouse stains (Table 4-2), 4D11 and K2D are believed to be specific for a non-polymorphic determinant on Ly-49G2 (Mason et al., 1989; Ortaldo et al., 1998; Kubota. 1997). In contrast, Cwy-3 and Ck1 seem to recognize a polymorphic epitope shared only by B6 and 129/J Ly-49G2.

Although Cwy-3 is capable of staining 129/J and B6x129/J F_1 Rag-$1^{-/-}$ A-LAKs, it gave a lower MFI compared to that of B6 and B6 Rag-$1^{-/-}$ derived A-LAKs. A reduction in Ck1 staining relative to B6 Rag-$1^{-/-}$ was also observed on the F_1 Rag-$1^{-/-}$ mice. The reasons for these observations are not entirely clear. One can speculate that both Cwy-3 and Ck1 might have a lower affinity to the allelic form of Ly-49G2 from 129/J compared to that of B6. Since the cDNA and amino acid sequence of Ly-49G2 of 129/J is not known, the degree of identity of Ly-49G2 between 129/J and B6 cannot be determined. Thus, whether amino acid differences between B6 and 129/J Ly-49G2, should they exist, have any influence on Cwy-3 and Ck1 recognition remains to be determined. In addition, it is also possible that A-LAK from 129/J has down regulated its Ly-49G2 expression. If this is the case, and based on the receptor calibration model proposed by Kärre and colleagues (Olsson et al., 1995; Olsson et al., 1997), this might suggest that there is a ligand for Ly-49G2 found in 129/J but not in B6 mice, which interacts with Ly-49G2 with high affinity. Since both B6 and 129/J are H-2^b mice, this might further imply that other nonclassical class I MHC found in 129/J could be the major ligand for Ly-49G2. Nevertheless, the possibility that the low expression level of Ly-$49G2^{129/J}$ could be due to it possessing a weaker promoter than that of Ly-$49G2^{B6}$ cannot be formally ruled out. The last possibility is that Cwy-3 does not recognize Ly-$49G2^{129/J}$, but instead recognizes a Ly-49G2-like molecule which generally has a lower expression level. However, since Cwy-3 and Ck1 seem to specifically recognize a polymorphic epitope on Ly-49G2 and the reduction of Cwy-3 staining is also seen in F_1 Rag-$1^{-/-}$ mice, this explanation is rather unlikely.

To re-examine the protective effect of H-2^d products against Ly-49G2$^+$ NK cell lysis, we sorted the B6 Rag-$1^{-/-}$ A-LAKs into Cwy-3^+ and Cwy-3^- populations and determined their lytic activities against WEHI-164. The data indicated that both Ly-49G2$^+$ and Ly-49G2$^-$ A-LAKs failed to lyse this particular tumor cell line. However, the presence of Cwy-3, Ck1 or 4D11 only resulted in modest enhanced lysis of this tumor cell line by the Cwy-3^+ population (Fig. 4-10, upper panel). This is in agreement with previously published studies, and suggests that H-2^d product(s), and probably D^d can inhibit NK cell functions through Ly-49G2. Nevertheless, caution has to be exercised when interpreting these data, since it is possible that there are other H-2^d inhibitory receptors that are predominantly expressed on this Cwy-3^+ population.

In contrast to a report using 4D11$^-$A1$^+$ A-LAKs (Mason et al., 1995), no reversal of WEHI-164 lysis was detected by Cwy-3^-A1$^+$ in the presence of A1. These seemingly contradictory observations can be explained by the fact that only a small subset of the Cwy-3^- were actually Ly-49A$^+$ (Fig. 4-3). Presumably the majority of the 4D11$^-$A1$^+$ population used by Mason et al. were Ly-49A$^+$. However, the nature of this NK cell subset has to be redefined, since 4D11 also reacts with Ly-49A and it is difficult to reconcile the failure of 4D11 to stain this A1$^+$ population (Mason et al., 1995). In order to perform a more detailed analysis of Ly-49G2 regulatory functions, it is clear that the Cwy-3^+ A-LAKs which express other Ly-49 receptors have to be removed. Nevertheless, with these new mAbs, Cwy-3 and Ck1, more insight should be gained in the study of various NK cell subsets.

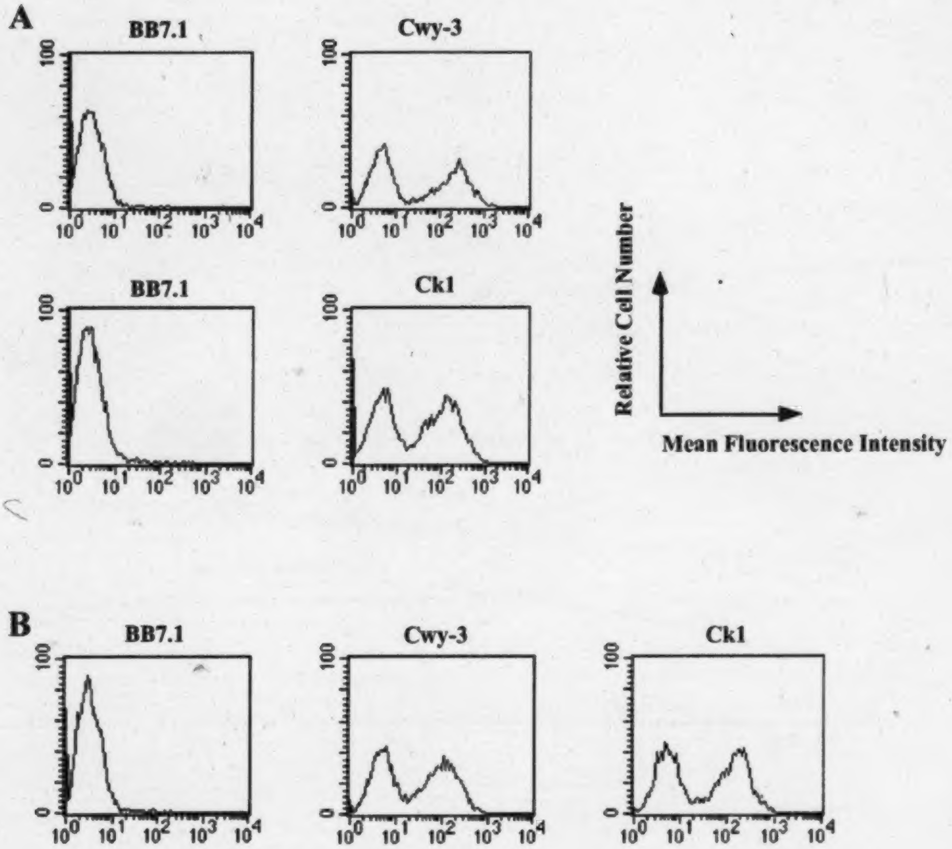


Figure 4-1. Expression of Cwy-3 and Ck1 defined cell surface marker(s) by B6 and B6 Rag-1$^{-/-}$ derived A-LAKs. (a). Half a million of B6 A-LAKs were stained with purified BB7.1 (as isotype control), purified Cwy-3 or Ck1 supernatant for 30 min at 4^{o}C, before being counter stained with FITC-conjugated rat anti-mouse IgG. Both Cwy-3 and Ck1 stained approximately 50% of the B6 A-LAKs. (b). Half a million of B6 Rag-1$^{-/-}$ A-LAKs were stained with Cwy-3 and Ck1. A similar percentage of Cwy-3^{+} and Ck1^{+} A-LAKs were detected in B6 Rag-1$^{-/-}$ A-LAKs as in normal B6 A-LAKs.

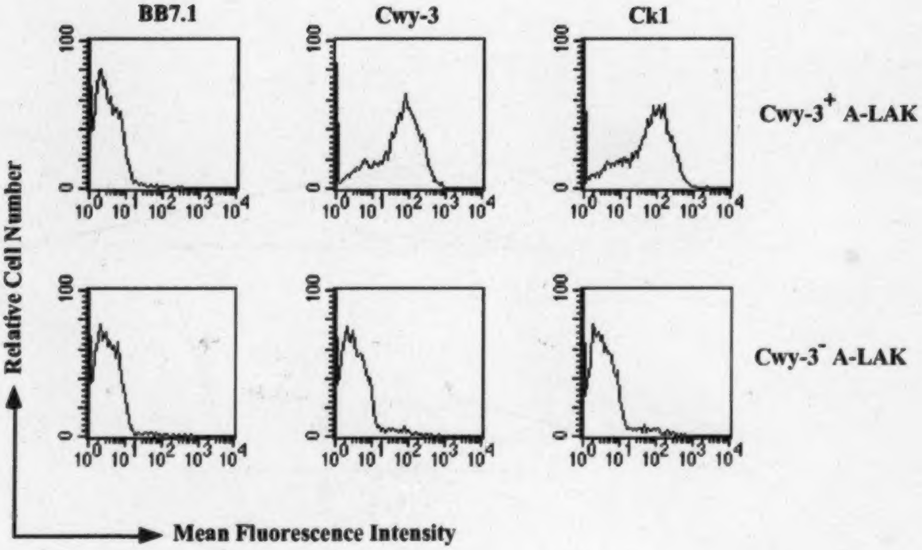


Figure 4-2. Sorting of B6 A-LAKs into Cwy-3$^+$ and Cwy-3$^-$ populations, and their expression of Ck1 defined cell surface marker. B6 A-LAKs were sorted into Cwy-3$^+$ and Cwy-3$^-$ populations by Dynabeads as described in *Materials and Methods*. After culture for three more days, the separated populations were harvested and re-analyzed for their expression of Cwy-3 and Ck1-specific markers. Both Cwy-3$^+$ and Cwy-3$^-$ populations were stained with BB7.1 (as isotype control), Cwy-3 or Ck1. The Cwy-3$^+$ and Cwy-3$^-$ populations remained stable for their expression of the Cwy-3 defined epitope after separation, and the staining pattern of Ck1 correlated with that of Cwy-3.

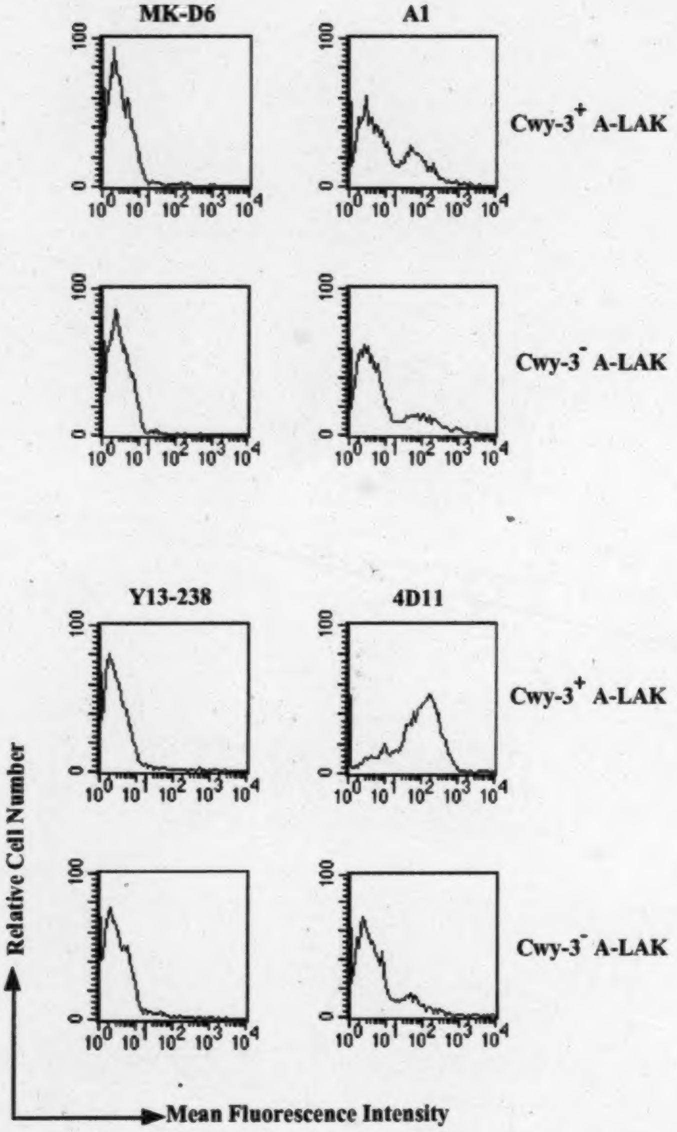


Figure 4-3. Expression of Ly-49A and G2 on B6 Cwy-3$^+$ and Cwy-3$^-$ A-LAKs. Magnetic bead separated Cwy-3$^+$ and Cwy-3$^-$ B6 A-LAKs were stained by MK-D6 (isotype control), A1, Y13-238 (isotype control) and 4D11. The staining patterns of both Cwy-3 and Ck1 on Cwy-3$^+$ and Cwy-3$^-$ B6 A-LAKs correlated with that of 4D11, but not with A1.

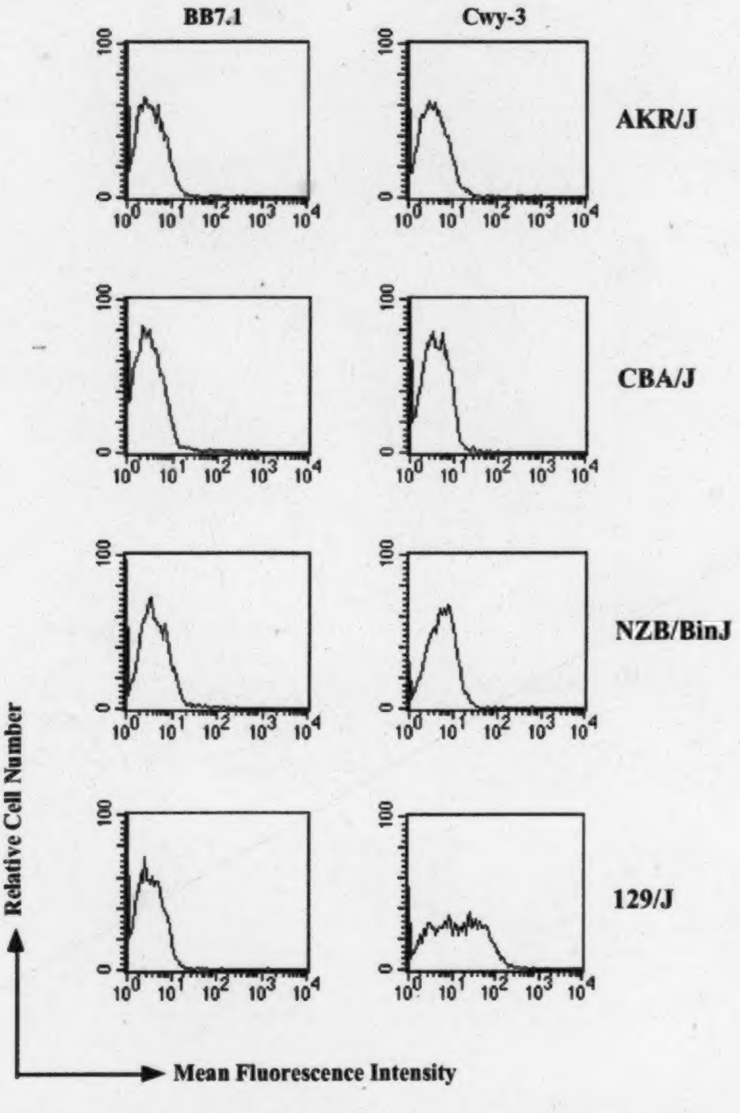


Figure 4-4. Cwy-3 stains A-LAKs derived from H-2^b mouse strains but not those derived from other H-2 haplotypes. Half a million A-LAKs generated from mouse strains with different H-2 haplotypes were stained with BB7.1 (as isotype control) or Cwy-3. Among the four mouse strains tested, only 129/J (H-2^b) A-LAKs stained positive by Cwy-3, but not those derived from AKR/J (H-2^k), CBA/J (H-2^k) and NZB/BinJ (H-2^d).

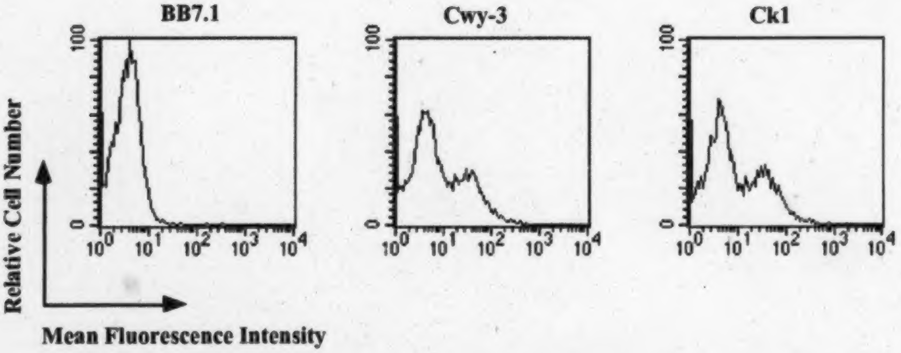


Figure 4-5. Reduction of the expression level of Cwy-3 and Ck1 defined epitope(s) on A-LAKs derived from B6x129/J F_1 Rag-1$^{-/-}$ mice. Half a million B6x129/J Rag-1$^{-/-}$ A-LAK were stained with BB7.1 (as isotype control), Cwy-3 or Ck1. Approximately forty percent of the A-LAKs stained positive by both Cwy-3 and Ck1.

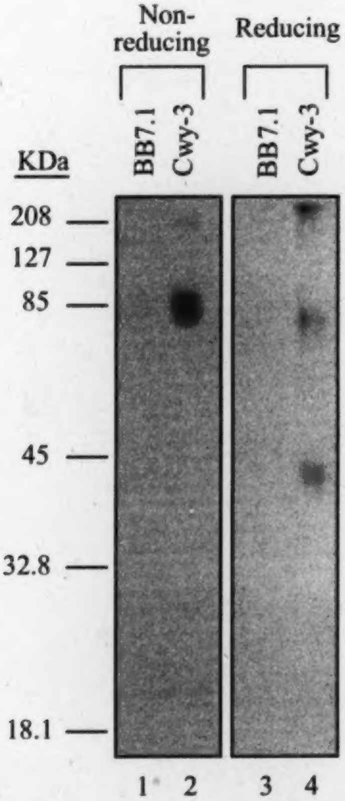

Non-reducing
Reducing
BB7.1
Cwy-3
BB7.1
Cwy-3
KDa
208
127
85
45
32.8
18.1
1
2
3
4

Figure 4-6. Immunoprecipitation of a B6 cell surface dimer by Cwy-3. The cell surface biotinylated B6 A-LAK cell lysates were immunoprecipitated by either BB7.1 (isotype control) or Cwy-3 as described in *Materials and Methods*. The immunoprecipitated proteins were then resolved on 10% SDS-PAGE under both nonreducing and reducing conditions, and visualized by ECL.

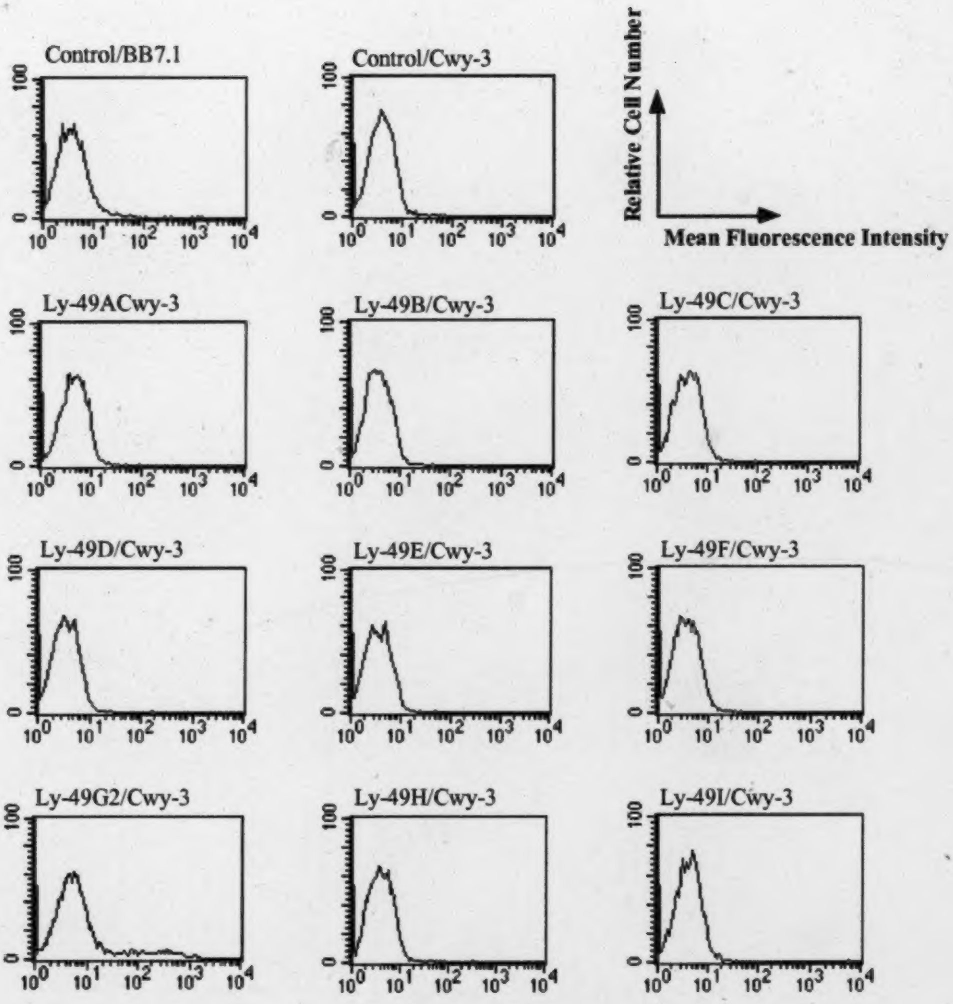


Figure 4-7. Cwy-3 stains Cos-7 cells transfected with Ly-49G2. Cos-7 was transfected with the pC1-neo expression vector containing cDNA encoding the B6 alleles of Ly-49A-I as described in *Materials and Methods*. At 48 h post-transfection, the cells were harvested, and stained with Cwy-3. Among all of the transfectants, only those Ly-49G2-expressing Cos-7 cells stained positive by this mAb.

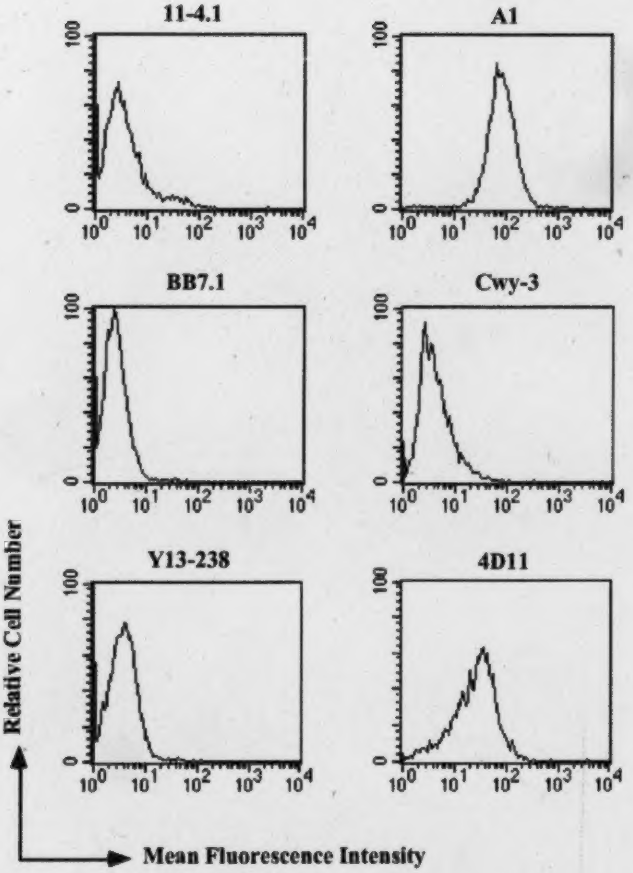


Figure 4-8. Expression of Ly-49A and G2 by EL-4. Half a million EL-4 T lymphoma cells were stained with 11-4.1 (as isotype control), A1, BB7.1 (as isotype control), Cwy-3, Y13-238 (as isotype control) and 4D11. EL-4 expressed extremely low levels of Ly-49G2 as detected by Cwy-3. However, a high level of Ly-49A expression was detected by both A1 and 4D11 staining.

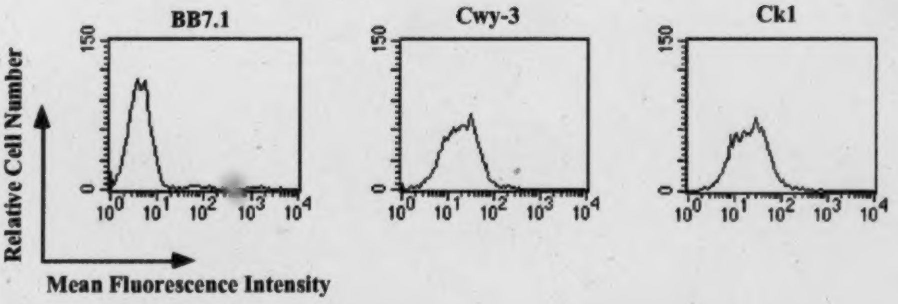


Figure 4-9. Cwy-3 and Ck1 stain EL-4/Cwy-3$^+$ to a similar extent. The Cwy-3 magnetic bead selected EL-4 subline was stained with either Cwy-3 or Ck1 by using BB7.1 as isotype control. The FACS analysis showed that both of these mAbs stained EL-4/Cwy-3$^+$ to the same extent.

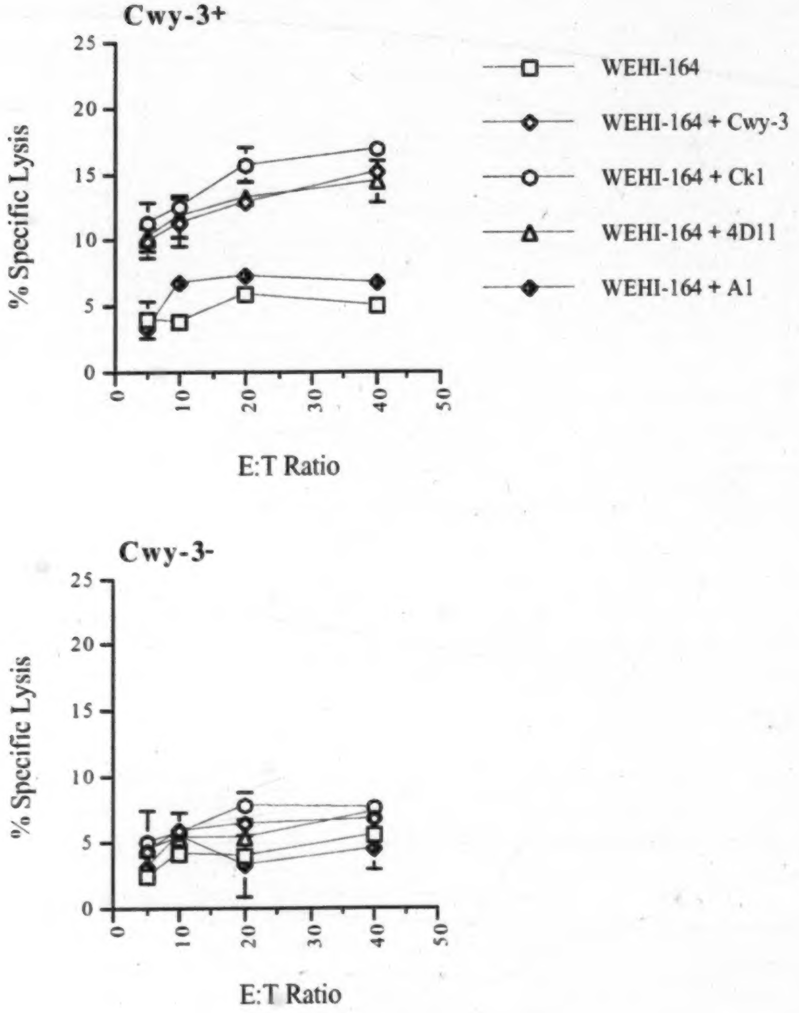


Figure 4-10. Cwy-3 and Ck1 increased the lysis level of WEHI-164 by B6 Cwy-3$^+$ but not Cwy-3$^-$ A-LAKs. Sorted B6 Cwy-3$^+$ and Cwy-3$^-$ A-LAKs were incubated with ^{51}Cr-labeled WEHI-164 in the presence of 20 μg/ml of the indicated mAbs at various E:T ratios for 4 h. Each E:T ratio was used for triplicate determinations (except the E:T ratio of 40:1 for Cwy-3$^+$ lysis of WEHI-164 in the presence of Ck1, which were carried only in duplicate) and the results are expressed as mean ± SD.

Table 4-1

Comparison of the distribution of 4D11, Cwy-3 and Ck1 defined epitopes on mouse strains with different H-2 haplotypes

Mouse Strains	4D11[a]	Cwy-3	Ck1
C57BL/6 (H-2^b)	+	+	+
129/J (H-2^b)	+	+	NA
Balb/c (H-2^d)	+	NA[b]	NA[b]
DBA/2 (H-2^d)	+	NA	NA
NZB/BinJ (H-2^d)	NA	-	NA
AKR (H-2^k)	+	-	-
CBA/J (H-2^k)	+	-	NA

[a] The mouse strain distribution of 4D11 was compiled from the data published by Mason et al., 1989. *J. Immunol.* 140:4403-4412 and Ortaldo et al., 1998. *J. Immunol.* 160:1158-1165.

[b] The staining of Balb/c A-LAKs by Cwy-3 and Ck1 has never been done. However, since these two mAbs are generated from Balb/c mice immunized with B6 A-LAKs, they are expected not to react with Ly-49G2$^{Balb/c}$.

[c] NA = Data not available.

+ = Positive staining; - = Negative staining

CHAPTER V

THE MURINE NONCLASSICAL CLASS I MAJOR HISTOCOMPATIBILITY COMPLEX-LIKE CD1.1 MOLECULE PROTECTS TARGET CELLS FROM LYMPHOKINE-ACTIVATED KILLER CELL CYTOLYSIS

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A. Introduction

Among all of the nonclassical class I MHC molecules, CD1 is the only functional antigen presenting molecule encoded outside the MHC locus (Shawar et al., 1994; Beckman and Brenner. 1995). The CD1 family itself is quite divergent, and based on amino acid sequence identity, human CD1 is classified into groups 1 and 2 (Calabi et al., 1989). The former group includes CD1a-c and e, whereas, group 2 only consists of CD1d (Shawar et al, 1994; Beckman and Brenner. 1995; Calabai et al., 1989). In mice, CD1 is encoded by two closely related genes, murine CD1.1 and CD1.2, that are most homologous to human CD1d (Shawar et al, 1994; Beckman and Brenner. 1995; Calabai et al., 1989). In contrast to human, no counterparts of human CD1a-c and e are found in the murine system.

Although classified as a class I MHC molecule, CD1 molecules share only 5-15% sequence identity with classical class I MHC molecules in the $\alpha 1$ and $\alpha 2$ domains. In the more conserved $\alpha 3$ extracellular domain, the sequence identity increases up to approximately 30%. In fact, CD1 molecules are about as closely related to class II MHC as they are to class I MHC molecules (Hughes. 1991), suggesting that they diverged relatively early in evolution from the MHC-encoded antigen presenting molecules. Comparison of X-ray crystallographic data has shown that mCD1.1 adopts a structure generally resembling both classical class I and class II MHC antigen presenting molecules, but with a narrower, deeper and more hydrophobic antigen binding groove (Zeng et al., 1997). Furthermore, like classical class I MHC molecules, it also co-crystallized with β_2m (Zeng et al., 1997).

Not only do CD1 molecules resemble both class I and II MHC molecules structurally, their assembly and trafficking pathways are a hybrid of both of these antigen presenting molecules. Like class I MHC, CD1 assembly and expression are β_2m but not invariant chain dependent (Brutkiewicz et al., 1995; Teitell et al., 1997). However, unlike class I MHC, its antigen presentation pathway is TAP-independent (Brutkiewicz et al., 1995; Teitell et al., 1997). This unusual behavior of CD1 is explained by its class II MHC-like intracellular trafficking due to an endosomal targeting signal in the cytoplasmic tail (Sugita et al., 1996), and the fact that CD1 binds glycolipids. Because of these unique characteristics, CD1 molecules are sometimes referred to as the third lineage of antigen presenting molecules.

It is now known that this third lineage of antigen presenting molecules are ligands for the TCR expressed by a subset of T cells also expressing NK cell markers, termed NKT cells (Bendelac et al., 1995). Furthermore, CD1 is capable of presenting hydrophobic antigens such as glycosylceramides and microbial lipoglycan to these T cells (Beckman et al., 1994; Sieling et al., 1995; Kawano et al., 1997; Burdin et al., 1998). The importance of CD1 endosome targeting becomes apparent, since the loading of these bacterial antigens actually takes place in the endosome (Prigozy et al., 1997; Jullien et al., 1997). However, it is also important to note that, in addition to hybrophobic glycolipids, CD1 has also been shown to present peptides with hybrophobic motifs (Castaño et al., 1995). These T cells secrete large amounts of IL-4 and IFN-γ upon activation by anti-CD3 mAb (Chen et al., 1997), or when these T cells are engaged by CD1 presenting bacterial antigens. It is believed that CD1 and NK T cells may be involved in determining Th1 and Th2 immune responses (Yoshimoto et al., 1995).

With the recent demonstration that NK cell cytotoxic activity can be regulated by nonclassical class I MHC molecules such as HLA-E and G (Braud et al., 1998; Borrego et al., 1998; Colonna et al., 1998b), and with the crystallographic and functional data regarding CD1 molecules, this prompted us to examine the ability of mCD1.1 to regulate NK cell functions. In this study, we present evidence that mCD1.1 negatively regulates murine NK cell cytotoxic activity. This is the first demonstration that a non-MHC locus encoded nonclassical class I MHC-like molecule can regulate NK cell functions. Furthermore, our data also suggest the existence of a conserved mCD1.1-specific inhibitory receptor(s).

B. Materials and Methods

Mice. Female C57BL/6 (B6, H-2^b) mice were obtained from the Jackson laboratory (Bar Harbor, ME) and the Charles River Laboratories (Wilmington, MA). Female CBA/J (H-2^k), NZB/BinJ (H-2^d) and B6x129/J F_1 Rag-$1^{-/-}$ (H-2^b) mice were purchased from the Jackson Laboratory. All mice were eight to twelve-week of age, except NZB/BinJ, which were nine months of age.

mAbs. 1B1 (rat IgG_{2b}), anti-mCD1, has been described previously (Brossay et al., 1997). M1/89.18.7.HK (rat IgG_{2b}), anti-CD45, and 7D2-1.4.1.5 (rat IgG_{2b}), anti-mouse IgG_{2a} and IgG_{2b}-specific hybridomas, were obtained from ATCC (Manassas, VA). Purified M1/89.18.7.HK was obtained by $(NH_4)_2SO_4$ precipitation and dialyzed against PBS. FITC-conjugated goat anti-rat IgG was obtained from Jackson ImmunoResearch Laboratory (West Grove, PA).

Tumor cell lines and cloned CTL. The NK cell sensitive target Yac-1 T lymphoma was obtained from ATCC (Manassas, VA). The RMA/S and RMA/S.CD1.1 transfectant have been described previously (Teitell et al., 1997). The RMA T lymphoma was a gift from Dr. W. Jefferies (University of British Columbia, Vancouver, Canada). All of the tumor cells line were maintained in RPMI supplemented with 5% heat-inactivated FCS, 20 mM Hepes, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. For RMA/S.CD1.1, 100 μg/ml of G418 was also added to the culture. Clone 3/4 is a C57BL/6-derived CTL clone specific for D^b and the influenza nucleoprotein peptide (NP) (365-380) of the A/PR/8/34 influenza virus (Kane and Mescher. 1993). The CTL clone was maintained as previously described (Kane and Mescher. 1993).

Immunofluorescence staining. Half a million tumor cells were first incubated with 10 μg/ml of 2.4G2 for half an hour and then washed. This was then followed by incubation with 100 μl of the 7D2-1.4.1.5 hybridoma supernatant or 2 μg of purified 1B1 for 30 min at 4°C. After extensive washing, the cells were stained with 100 μl of 1:100 dilution of FITC-coupled goat anti-rat IgG secondary antibody for 30 min at 4°C. The samples were then analyzed by FACScan (Becton Dickinson, Mountain View, CA).

Generation of poly I:C-activated killer cells. Each B6 mouse was injected i.p. with 200 μg of poly I:C. After 18 h, spleen cells were harvested from these mice and RBCs were removed by lysing with 0.14 M NH_4Cl. Adherent cells were then removed by culturing the splenocytes on tissue culture-treated plate for 1 h at a density of 5×10^6 cells/ml at 37°C. The non-adherent cells were then used as effectors in ^{51}Cr release assays.

Generation of adherent lymphokine-activated killers (A-LAKs). A-LAKs from different mouse strains were generated as previously described (Gunji et al., 1989). Briefly, adherent cells from the total splenocyte population were removed by culturing them on tissue culture-treated plates for 1 h. Non-adherent cells were then harvested and passed through a nylon wool column to remove B cells. The NWNA cells, at a density of $1\text{x}10^6$ cells/ml, were cultured in RPMI/10% FCS supplemented with sodium pyruvate, non-essential amino acids, $5\text{x}10^{-5}$ M 2-ME (herein referred to as complete medium) and with 800 units/ml of recombinant human IL-2 (rIL-2) expressed in and purified from *E. coli.* After 3 days of culture, the non-adherent LAKs (NA-LAKs) were removed, and the adherent LAKs (A-LAKs) were then maintained in complete RPMI/10% FCS with 800 units/ml of rIL-2 for four more days. Operationally, these A-LAKs were referred to as day 3 A-LAKs. For the NA-LAKs, they were recultured an additional day in the presence of the same concentration of rIL-2, and the A-LAKs generated from this culturing condition were referred to as day 4 A-LAKs. After the 1 day of culture, the NA-LAKs generated from the day 4 A-LAKs were removed, and recultured as before and the A-LAKs obtained were referred to as day 5 A-LAKs. The process was repeated until day 7 A-LAKs were obtained. In all instances, only the A-LAK were used as effectors in ^{51}Cr release assays. For 129xB6 F_1 Rag-$1^{-/-}$ mice, A-LAKs were obtained by culturing the RBC-depleted splenocytes with 800 units/ml of rIL-2 for 6 days. The NA-LAKs were then removed, and the A-LAKs were culture for 6 more days in the presence of rIL-2.

Cytotoxic and mAbs blocking assays. Targets cells were labeled with ^{51}Cr for 1 h. After extensive washing, $1\text{x}10^4$ target cells were incubated with poly I:C-activated killer or A-LAKs at various E:T ratios for 4-5 h at 37°C in a V-bottom microtitre plate in triplicate. For killing by CTL clone 3/4, RMA/S and RMA/S.CD1.1 were incubated at 26°C overnight to induce higher expression of D^b. The cells were then labeled with ^{51}Cr and pulsed with 400 μg/ml of NP peptide, YASNENMETM, at 37°C for 1 h prior to the cytotoxic assay. For mAb blocking assay, the ^{51}Cr-labeled targets were incubated with 40 μg/ml of M1/89.18.7.HK or 1B1 for 30 min at room temperature. The effectors were then added to the wells diluting the mAbs to give a final concentration of 20 μg/ml. The ^{51}Cr release assay was carried out in the presence of these mAbs for 4-5 h at 37°C. In all instances, the percentage lysis was determined as [(experimental release - spontaneous release)/(maximum release - spontaneous release)] x 100. Unless stated otherwise, all cytotoxic assays in this report were performed in triplicate.

C. Results

mCD1.1 expression inhibits A-LAKs cytotoxic activity. We were interested in examining the ability of mCD1.1 to inhibit NK cell cytotoxic activity. To address this issue, we took advantage of the fact that mCD1.1 expression is TAP-independent (Brutkiewicz et al., 1995; Teitell et al., 1997), and thus mCD1.1 can be expressed on the cell surface by the NK cell sensitive TAP-2 deficient T lymphoma cell line, RMA/S (Teitell et al., 1997). As detected by the mCD1.1-specific mAb 1B1, RMA/S expressed high levels of mCD1.1 upon transfection compared to that of the untransfected RMA/S control (Fig. 5-1). Since classical class I MHC expression requires the presence of functional TAP, this system enables us to assess the potential protective effect of mCD1.1 without the complication of inhibition also mediated by the endogenous classical class I MHC products D^b and K^b.

We first carried out an *in vitro* killing assay of RMA/S and RMA/S.CD1.1, using poly I:C-activated B6 NK cells as effectors. As expected, the class I MHC deficient cell line, RMA/S, was susceptible to NK cell lysis. However, a modest but reproducible reduction in lysis of the RMA/S.CD1.1 transfectant was detected at high E:T ratios where cytotoxicity was substantial, but apparently not plateau, and could be readily compared with the experimental groups (Fig. 5-2). This suggested that mCD1.1 is able to inhibit NK cell cytotoxic activity, but perhaps only a small fraction of the total poly I:C-activated NK cells population is negatively regulated by mCD1.1. Therefore, we performed cytotoxic assays using B6 A-LAKs to test the possibility that mCD1.1 regulated killer cells could be expanded when cultured in the presence of rIL-2. RMA/S and the RMA/S.CD1.1 transfectant were compared for their sensitivity to lysis by NWNA B6 spleen cells that become plate adherent in succeeding days of culture in rIL-2. Both RMA/S and the RMA/S.CD1.1 transfectant were highly susceptible to lysis by the day 3 and 4 A-LAKs. Nevertheless, a modest reduction in lysis of RMA/S.CD1.1 by these A-LAKs was detected (Fig. 5-3). Interestingly, day 5 and 6 A-LAKs cytotoxic activities against the RMA/S.CD1.1 transfectant were substantially reduced relative to the highly sensitive RMA/S target cell (Fig. 5-3). This pattern of resistance to A-LAK lysis generated at later time points was also detected with another independent RMA/S.CD1.1 clone (Fig. 5-4). Taken together, the preceding results obtained with rIL-2-activated A-LAKs strongly argue that mCD1.1, like classical class I MHC, may negatively regulate A-LAK cytotoxic activity. In agreement with the poly I:C data, it seems that a small proportion of the initial A-LAK population is negatively regulated by mCD1.1, which possibly can be expanded by passaging and culturing in the presence of rIL-2.

mCD1.1 inhibits NK cell cytotoxicity to the same extent as classical class I MHC molecules. The A-LAK mediated cytotoxicity data demonstrated the inhibitory effect of mCD1.1. However, it did not show its inhibitory efficiency compared to that of classical class I MHC. To address this issue, the level of RMA/S.CD1.1 lysis by pooled day 5 and 6 B6 A-LAKs was compared with that of RMA (from which RMA/S is derived). The class I MHC-positive cell line, RMA, was relatively resistant to day 5 and 6 A-LAKs lysis even at high E:T ratios (Fig. 5-5), whereas, RMA/S was lysed to a similar degree as the NK cell sensitive target T lymphoma, Yac-1. In contrast, RMA/S.CD1.1 was as resistant to day 5 and 6 A-LAK cytotoxic activity as RMA (Fig. 5-5). Thus, these results suggest that mCD1.1 can negatively regulate NK cell cytotoxity to the same extent as classical class I MHC. Since the day 5 and 6 B6 A-LAKs failed to lyse both RMA and

RMA/S.CD1.1, this further suggests that these A-LAKs can be negatively regulated by both classical class I MHC molecules and mCD1.1.

RMA/S.CD1.1 transfectant remains sensitive to cell-mediated cytotoxicity. It is not unreasonable to argue that upon mCD1.1 transfection the intrinsic properties of the cells had changed, and the transfectant simply becomes resistant to cell-mediated cytotoxicity, independent of killer cell recognition events. However, this interpretation is not supported by the observation that both RMA/S and RMA/S.CD1.1 were lysed to a similar degree by A-LAKs generated from early time points (Fig. 5-3). In addition, following induction of higher classical class I MHC expression at 26°C and incubated with influenza NP peptide antigen, both RMA/S and the RMA/S.CD1.1 transfectant were lysed to a similar high level by the influenza NP-specific, D^b restricted clone 3/4 (Fig. 5-6). For instance, only after pulsing with D^b-specific influenza NP peptide, but not in the absence of peptide could lysis be detected. This indicated that the intracellular apoptotic pathways remain intact upon mCD1.1 transfection. Thus, RMA/S.CD1.1 is not intrinsically resistant to cell-mediated lysis.

NK cell cytotoxic activity is inhibited by mCD1.1 expression. Our results obtained with A-LAK did not directly address whether mCD1.1 inhibited the cytotoxic activity of the NK cells. To this end, we directly addressed this issue by using B6x129 F_1 Rag-$1^{-/-}$ mice. Since the recombination activator gene, Rag-1, is critical in V(D)J recombination in both B and T cells, Rag-$1^{-/-}$ knock out mice do not produce any mature B and T lymphocytes (Mombaerts et al., 1992). As a result, the A-LAKs generated from Rag-$1^{-/-}$ will not contain CTLs or NKTs, and thus no cell-mediated cytolysis will be contributed by these populations. As showed in Fig. 5-7, RMA/S.CD1.1 but not RMA/S was resistant to lysis by B6x129/J F_1 Rag-$1^{-/-}$ A-LAK cells. Since no effector T cells are present in the system, we conclude that NK cell cytotoxic activity against the NK cell sensitive RMA/S target cell can be inhibited by mCD1.1 expression.

mCD1.1 inhibits A-LAK cytotoxic activity generated from mouse strains of different H-2 haplotypes. Since CD1 is a non-polymorphic class I MHC-like molecule, this implies that any NK cell receptor(s) which may mediate negative regulatory signals upon mCD1.1 engagement could be conserved in mice. Thus, mCD1.1 may be able to inhibit the cytotoxic activities of A-LAK derived from different inbred mouse strains. To test this hypothesis, we generated A-LAKs from two additional mouse strains, CBA/J (H-2^k) and NZB/BinJ (H-2^d). The results indicated that the A-LAKs from these mouse strains exhibited similar patterns of mCD1.1-mediated inhibition as seen with B6 A-LAKs (Fig. 5-8A and B). For instance, the day 3 and 4 A-LAKs lysed both RMA/S and RMA/S.CD1.1 efficiently, whereas, a significant reduction in lysis of RMA/S.CD1.1 was detected if day 5, 6 and 7 A-LAKs were used as effectors. It is perhaps worth noting that the patterns of inhibition among these mice are not exactly identical. In both B6 and NZB/BinJ mice, RMA/S was highly susceptible to all A-LAKs lysis, and the reduction of RMA/S.CD1.1 became clear in those A-LAKs generated at later time points (Fig. 5-3 and Fig. 5-8B). In contrast, A-LAKs derived from CBA/J showed a somewhat different kinetic pattern. The day 3 and 4 A-LAKs have low cytotoxicity against RMA/S, and it only became strongly observable with the day 6 and 7 A-LAKs (Fig. 5-8A), whereas RMA/S.CD1.1 remained relatively resistant to A-LAK obtained at all of the various culture periods with rIL-2 (Fig. 5-8A). The reasons for these somewhat different kinetic inhibition patterns are not clear. Nevertheless, these results demonstrate that mCD1.1 is able to suppress A-LAK activities of different mouse strains expressing distinct H-2 haplotypes, consistent with the possibility that a conserved NK cell inhibitory receptor(s) for mCD1.1 may exist that is able to suppress A-LAK cytotoxic activity.

An anti-mCD1.1 mAb, 1B1, partially restores the A-LAKs lysis of RMA/S.CD1.1. The preceding *in vitro* cytotoxic assays suggest that mCD1.1 is capable of negatively regulating A-LAK cytotoxic activity. To confirm this, we tested whether the inhibition of lysis of RMA/S.CD1.1 target could be reversed in the presence of an antibody that recognizes mCD1.1. Consistent with the previous results, reduction of A-LAK-mediated lysis of RMA/S.CD1.1, relative to RMA/S, was observed in the absence of any mAb (Fig. 5-9). However, with a final concentration of 20 μg/ml of 1B1, lysis of RMA/S.CD1.1 was partially restored (Fig. 5-9). This restoration was not mediated by antibody-dependent cell-mediated cytotoxicity (ADCC), since in the presence of an isotype control mAb, M1/89.18.7.HK specific for CD45, no restoration of lysis was detected (Fig. 5-9). It is important to note that both RMA/S and RMA/S.CD1.1 expressed the same level of CD45 as detected by M1/89.18.7.HK (data not shown). Therefore, these data indicate that cell surface expression of mCD1.1 is likely to be critical in inhibiting the A-LAKs cytotoxic activity. In all attempts with the 1B1 antibody, we were unable to restore the killing of RMA/S.CD1.1 to the same level as that of RMA/S. The reason for this incomplete restoration of lysis by 1B1 is not clear. It is perhaps interesting to note that 1B1 also only partially inhibits peptide specific and mCD1.1-restricted CTL lysis (Lee et al., 1998). It is possible that 1B1 recognizes an epitope that is only partially overlapping with the putative receptor(s) which mediates the inhibition of A-LAK lysis.

D. Discussion

In this study, we have shown that target cells are protected from A-LAK/NK cell cytotoxic function by expression of mCD1.1. We first observed that mCD1.1 expression protected target cells against the cytotoxicity of poly I:C-activated NK cells only at high E:T ratios. There are at least two possible explanations for this observation: that only a small fraction of the total NK cell population expresses the inhibitory receptor(s) for mCD1.1, or that only this population expresses the relevant receptor(s) at high enough density to deliver the inhibitory signals. Regardless of the true nature of this population, it or a subset with similar properties can be detected by consecutively transferring NA-LAKs early in culture in rIL-2 to generate A-LAKs later in culture. Whether this protocol actually expands a relevant but small NK cell subpopulation, or it simply induces upregulated expression of an inhibitory receptor(s) for mCD1.1, remains to be determined.

The mCD1.1 protein is by far the most divergent class I MHC molecule demonstrated to inhibit NK cell cytotoxic activity. However, it has been reported that human CD1a, b and c do not protect target cells from NK cell lysis (Storkus et al., 1996). It is important to note that these group 1 CD1 molecules have rather different amino acid sequences than that of group 2 CD1 molecules, which includes human CD1d, mCD1.1 and 1.2. For instance, the $\alpha1$ and $\alpha2$ domains of human CD1d are more similar to that of mCD1.1 and 1.2 than they are to the other human CD1 molecules (Calabai et al., 1989). These differences might explain why human CD1a, b and c failed to inhibit NK cell cytotoxic functions. Therefore, it will be interesting to determine whether human CD1d also protects target cells from lysis by NK cells. Another major difference between the human CD1 study and ours, is that the NK cells used earlier were isolated from peripheral blood, and in some instances were further activated by rIL-2 for only 18 h (Storkus et al., 1996). It is possible that this short term culturing condition did not allow the up-regulation of an inhibitory receptor(s) specific for CD1, out growth of relevant cell subpopulations, or other changes that would result in CD1-mediated protection of target cells. It can be further speculated that the cell line used in the human study can also contribute to the failure of human CD1 to inhibit NK cell functions. For instance, the use of C1R transfected with human CD1 might not possess the appropriate glycolipids that are required for the inhibitory effect. This assumes, of course, that the inhibitory effect of CD1 is antigen dependent.

Murine CD1 is encoded by two highly related genes, mCD1.1 and mCD1.2, which are resulting from gene duplication. Amino acid sequence analysis reveals that mCD1.1

and mCD1.2 share more than 95% identity (Shawar et al., 1994; Beckman and Brenner. 1995). However, based on the presence of mRNAs, their expression levels are rather different. For instance, mCD1.1 is highly expressed in all tissues compared to mCD1.2, except in thymus where both mCD1 genes are expressed to approximately the same levels (Bradbury et al., 1988). In addition, mCD1.2 lacks a conserved cys residue in its $\alpha2$ domain, which is presumably important in forming a disulfide bridge in mCD1.1 and other class I MHC molecules (Bradbury et al., 1988). Thus, mCD1.2 has been suggested to be non-functional. Whether mCD1.2 can inhibit NK cell cytotoxic activity similar to mCD1.1 remains to be determined. Interestingly, it is reported that B6 mice do not express mCD1.2, due to a frameshift mutation in exon 4 encoding for the $\alpha3$ domain (Park et al., 1998). The significance of this is not clear, but it raises the possibility that a deficiency in mCD1.2 expression might influence the expression level of putative inhibitory receptor(s) for mCD1.1, as can occur with NK cell inhibitory receptors specific for classical class I MHC molecules (Salcedo et al., 1997). For instance, with the lower level of mCD1 in B6 mice, these NK cells could be more sensitive to a slight change in mCD1 expression level.

The TAP-2 deficient cell line, RMA/S, can present peptides derived from cytosolic proteins on classical class I MHC through a TAP-independent pathway (Esquivel et al., 1992; Hosken and Bevan. 1992). Thus, it can be argued that the protective effect seen in RMA/S.CD1.1 is actually mediated by classical class I MHC and mCD1.1 only indirectly. For instance, mCD1.1-derived peptides might be bound to and stabilize the normally empty and thermally unstable D^b and K^b molecules expressed by RMA/S. This in turn would protect the target cells from A-LAK lysis. However, this argument is not supported by the fact that no increase in cell surface expression of D^b and K^b can be detected in the mCD1.1 transfectant (Teitell et al., 1997 and data not shown). Regulation of NK cell cytotoxicity by human nonclassical class I HLA-E has been described recently (Braud et al., 1998; Borrego et al., 1998). It was demonstrated that HLA-E presentation of leader sequences derived from other HLA is critical in this HLA-E mediated inhibition, through interaction with the CD94/NKG2A heterodimer (Braud et al., 1998; Borrego et al., 1998). Since murine nonclassical class I Qa-1^b is the mouse orthologue of HLA-E, it is possible that the mCD1.1-dependent inhibition of A-LAK cytotoxic activity is actually mediated by Qa-1^b. However, this interpretation is highly unlikely, since comparison of the leader sequence of mCD1.1 with those bound by Qa-1^b, shows that the signal peptide of mCD1.1 does not contain the necessary motif to bind Qa-1^b (Bradury et al., 1998; Soloski et al., 1995). An additional argument against such interpretation of the data is that a mCD1.1-specific mAb substantially restored the susceptibility of RMA/S.CD1.1 to NK cell lysis. This indicated that the suppression is indeed mediated by intact, cell surface mCD1.1. It has been previously documented that untransfected RMA/S cells do express some cell surface mCD1 (Brossay et al., 1997). The amount of mCD1 expression by these cells is much less than the expression level in RMA/S.CD1.1 transfectants. However, the level of mCD1.1 expression in RMA/S cells supports a response to lipoglycan α-galactosylceramide, but is insufficient for stimulating peptide-specific T cells (Burdin et al., 1998), in addition to not inhibiting NK cell activity. However, it should be noted that the level of mCD1.1 expression on the RMA/S transfectant is not unphysiologic, and is similar to the level present on B cells and freshly isolated dendritic cells (Park et al., 1998; Brossay et al., 1998).

Murine CD1.1 is non-polymorphic (Shawar et al., 1994; Beckman and Brenner. 1995) and we observed that its expression protected RMA/S from lysis by A-LAKs from a variety of mouse strains. The conserved structure of mCD1.1 and the broad pattern of A-LAK inhibition suggests the existence of a conserved inhibitory receptor(s) specific for mCD1.1 expressed by A-LAK cells of the strains of mice we have tested. It is unlikely that the inhibition is mediated by NK cell inhibitory receptors Ly-49A, G or C/I, since these molecules are detected on B6 day 3 and 4 A-LAKs, and RMA/S.CD1.1 is highly susceptible to lysis by these A-LAKs. Based on the inhibition pattern of mCD1.1, one would predict that the receptor(s) would be absent or reduced in expression on day 3 and 4

A-LAKs but presence on A-LAKs from later time points. Since no mAbs specific for other potential Ly-49 inhibitory receptor family members such as Ly-49B, E and F have been described, we do not know the expression pattern of these molecules on A-LAKs generated at different time points. It remains possible that Ly-49 members with divergent amino acid sequences, such as Ly-49B, might recognize mCD1.1. However, it has been demonstrated that inhibition of NK cell function by class I MHC molecules can be achieved in the absence of Ly-49 receptors expression (Williams et al., 1997; Sivakumar et al., 1997; Manoussaka et al., 1998). This is especially true for NK cells derived from fetal tissues (Williams et al., 1997; Sivakumar et al., 1997; Manoussaka et al., 1998). Interestingly, Kumar and colleagues have suggested that these Ly-49$^-$ NK cells might be regulated by nonclassical class I MHC molecules (Sivakumar et al., 1997). Nevertheless, the suggestion of a lectin-like inhibitory receptor for mCD1.1 is rather interesting especially since mCD1.1 possesses five potential *N*-linked glycosylation sites on its extracellular domains (Bradbury et al., 1988). With the recent cloning of murine CD94 and NKG2 (Vance et al., 1997; Vance et al., 1998, Lohwasser et al., 1999; Silver et al., 1999), CD94/NKG2 heterodimers can potentially be the receptor(s) for mCD1.1. However, mAbs specific for these receptors have to be developed to assess their involvement in mCD1.1-mediated inhibition. Nevertheless, with the demonstration that CD94/NKG2A heterodimers only recognize nonclassical class I MHC Qa-1^b presenting signal peptide derived from other class I MHC molecules (Vance et al., 1998), this makes CD94/NKG2 NK cells receptors unlikely to be the candidate for mCD1.1-specific inhibitory receptor(s). Another candidate for a mCD1.1-specific inhibitory receptor(s) is gp49B1, which belongs to the Ig superfamily (Wang et al., 1997). However, this inhibitory receptor is expressed on all NK cells, and can be detected on A-LAKs which have been cultured for 3 days (Wang et al., 1997). Thus, it seems unlikely that gp49B1 is the inhibitory receptor(s) for mCD1.1. It is reported that another NK cell inhibitory receptor, MAFA (2F1), expression is upregulated by culturing in the presence of IL-2 (Hanke et al., 1998). Thus, it is possible that mCD1.1 can interact with this particular receptor, but this hypothesis remains to be tested. At present, the identity of the mCD1.1-specific inhibitory receptor(s) remains elusive. However, identification of this inhibitory receptor(s) should aid in the understanding of regulatory mechanisms of NK cells.

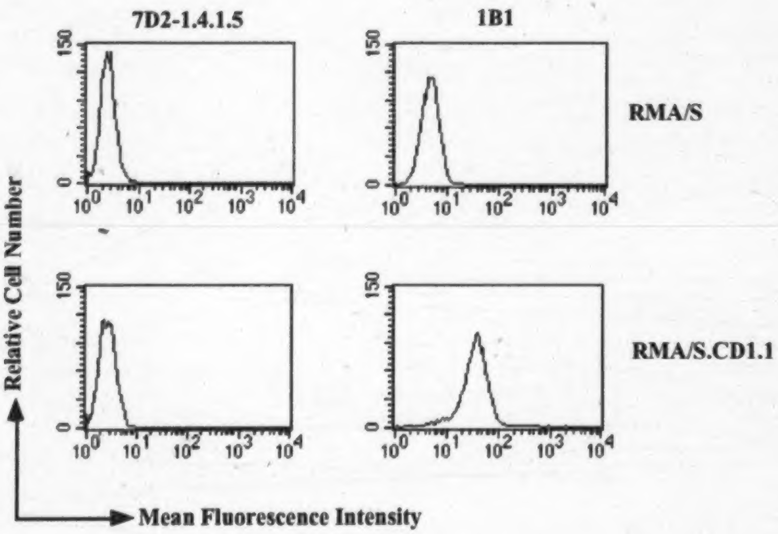


Figure 5-1. Expression of mCD1.1 by RMA/S and RMA/S.CD1.1 transfectant. Half a million cells were stained with 7D2-1.4.1.5 supernatant or 2.5 μg purified 1B1 for 30 min at 4°C. The cells were then washed and counter stained with FITC-conjugated rat-anti mouse IgG.

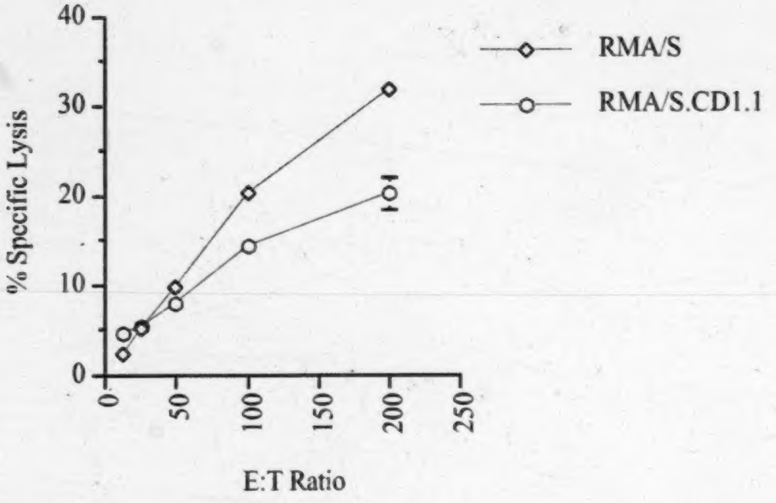


Figure 5-2. mCD1.1 partially inhibits the cytotoxic activity of poly I:C-activated B6 NK cells at high E:T ratios. Female B6 mice were injected with 200 μg of poly I:C, and 18 h later the spleen cells were harvested and the plastic non-adherent spleen cells were then used as effectors for the lysis of both ^{51}Cr-labeled RMA/S and RMA/S.CD1.1. Each E:T ratio was used for triplicate determinations, and the results are expressed as mean ± SD. All spontaneous release values were <10.3 %.

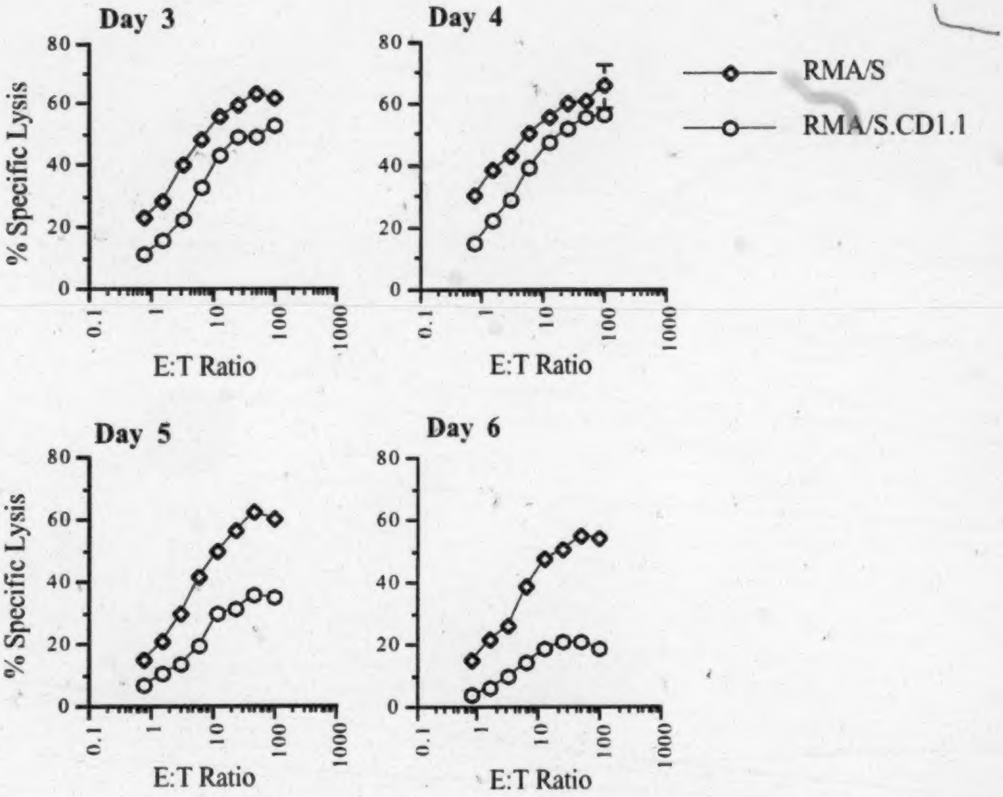


Figure 5-3. The extent of mCD1.1-mediated inhibition of B6 A-LAK cytotoxic activity is affected by passage of LAKs. B6 A-LAKs were generated by daily transfer of NA-LAKs to new flasks as described in *Materials and Methods*. Lysis of the RMA/S.CD1.1 transfectant was compared with the parental cell line, RMA/S. The ^{51}Cr-labeled targets were then incubated with the A-LAKs from all groups obtained on day 7 of culture at different E:T ratios at 37°C for 4 h. Results are expressed as mean of triplicate wells ± SD.

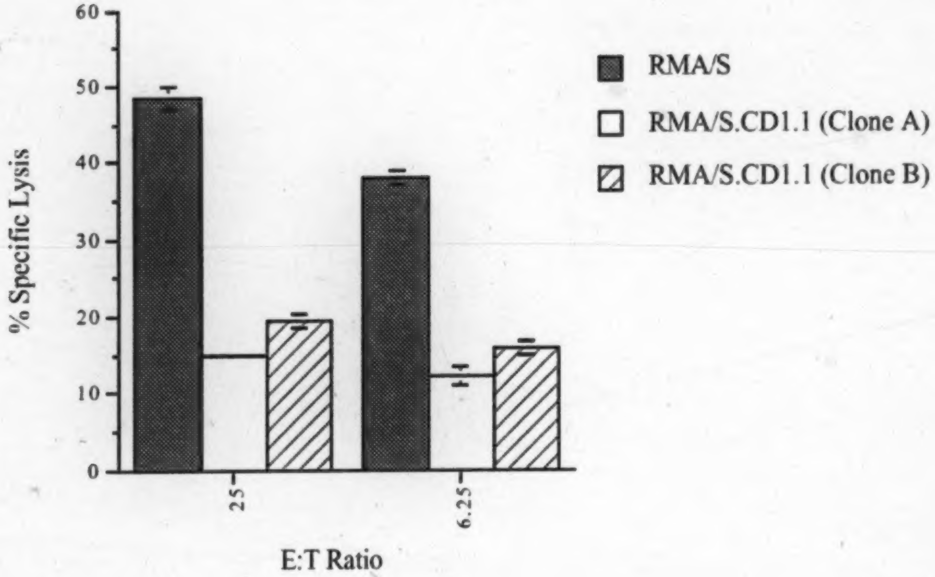


Figure 5-4. Two independent clones of RMA/S.CD1.1 transfectant exhibit similar resistance from the cytotoxic activity of pooled day 5 and 6 B6 A-LAKs. The ^{51}Cr-labeled RMA/S.CD1.1 clones (A and B) resist lysis by B6 A-LAKs, generated at later time points, to a similar extent. The RMA/S.CD1.1 (clone A) is the transfectant used throughout this study. The cytotoxic assay was carried out for 4 h at 37°C. Each E:T ratio was used for triplicate determinations, and the results are shown as mean of triplicate wells ± SD.

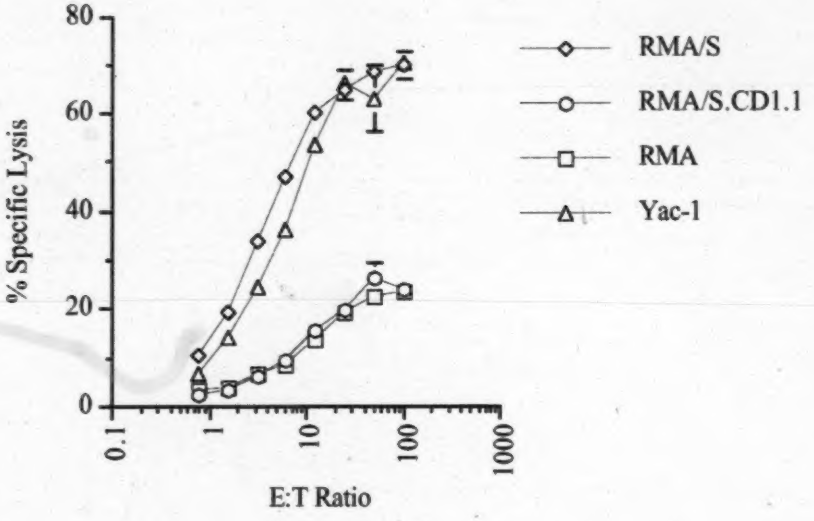


Figure 5-5. Expression of mCD1.1 renders RMA/S as resistant to pooled day 5 and 6 A-LAKs as the classical class I MHC-expressing RMA cell line. Ten thousand ^{51}Cr-label target cells were incubated with pooled day 5 and 6 B6 A-LAKs for 4 h at 37°C. Both RMA/S and Yac-1, but not RMA and RMA/S.CD1.1, were extremely sensitive to the cytotoxic activity of these A-LAKs. Each data point represents triplicate determinations, and data are shown as mean ± SD. All spontaneous release values were < 17.9%.

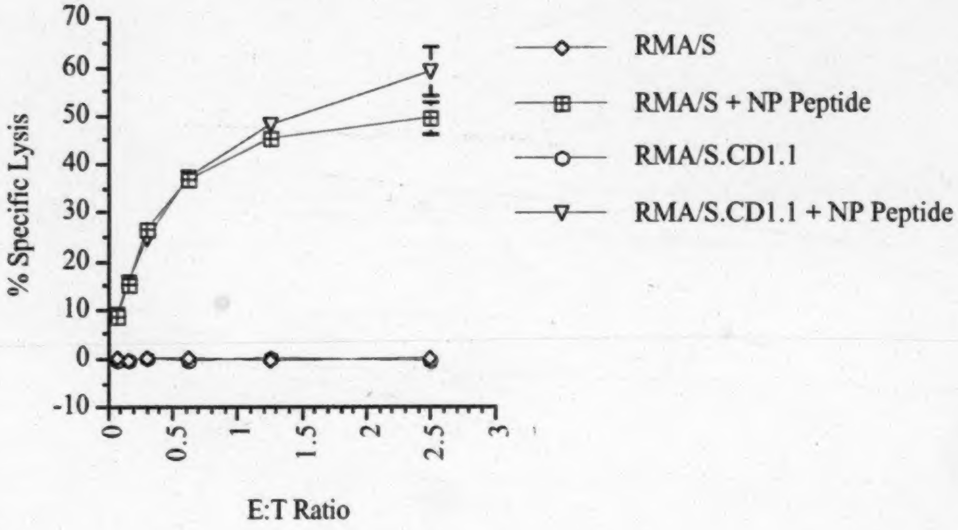


Figure 5-6. The mCD1.1 transfectant of RMA/S is not intrinsically resistant to cell-mediated lysis. RMA/S and RMA/S.CD1.1 cells were grown overnight at 26°C to induced higher expression of D^b before labeling with ^{51}Cr. These labeled targets were then pulsed with or without 400 μg/ml of NP peptide (YASNEMETM) at 37°C for 1 h and then washed. These target cells were then incubated with the 3/4 CTL clone at various E:T ratios in triplicate, and the assay was performed at 37°C for 4 h. Results are shown as mean ± SD, and the spontaneous release values were < 9.4%.

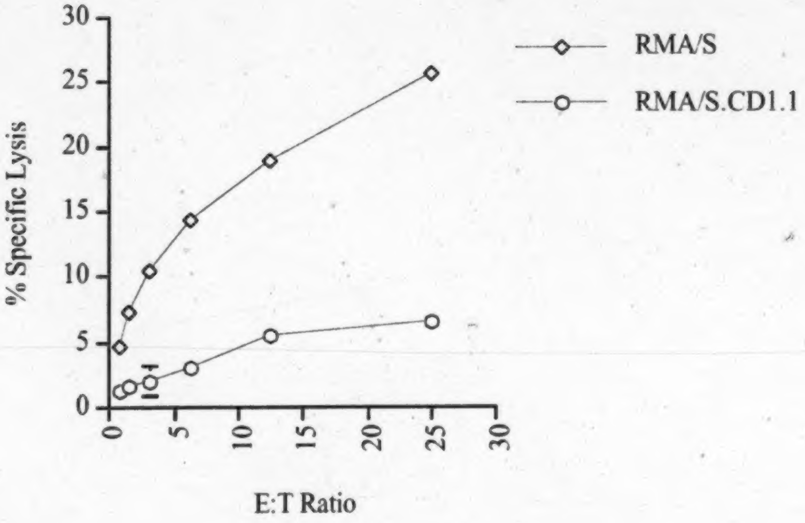


Figure 5-7. A-LAKs derived from 129xB6 F_1 Rag-1$^{-/-}$ mice lyse RMA/S, but not the RMA/S.CD1.1 transfectant. ^{51}Cr-labeled RMA/S and RMA/S.CD1.1 were incubated, at different E:T ratios at 37°C for 4 h, with A-LAKs generated from B6x129/J F_1 Rag-1$^{-/-}$ mice as described in *Materials and Methods*. Each E:T ratio was used for triplicate determinations, and the data are expressed as means ± SD. All spontaneous release values were < 9.6%.

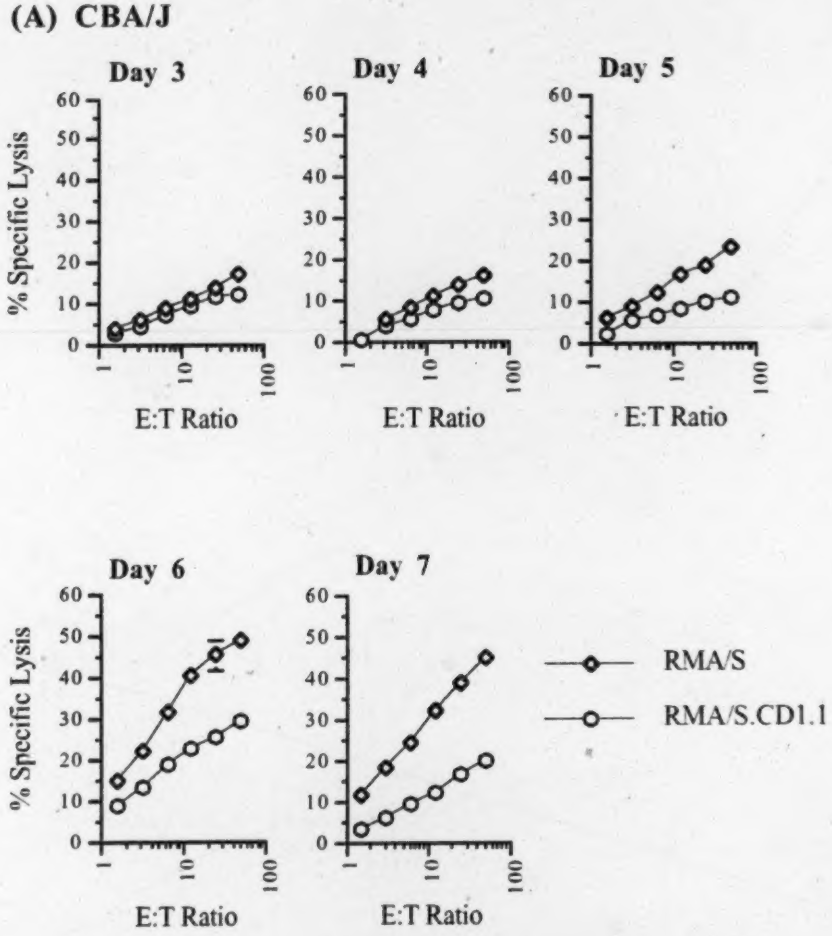
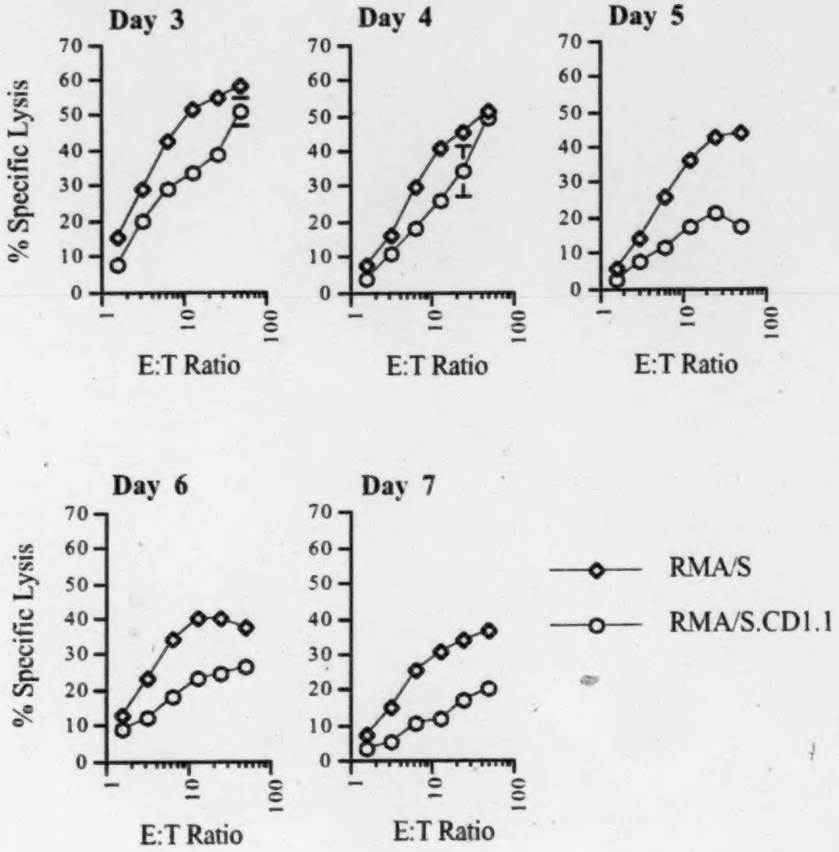


Figure 5-8. Expression of mCD1.1 inhibits the cytotoxic activity A-LAKs derived from mouse strains with different H-2 haplotypes. A-LAKs derived from (A) CBA/J (H-2^b) and (B) NZB/BinJ (H-2^d) were generated as described in *Materials and Methods*. The ^{51}Cr-labeled RMA/S and RMA/S.CD1.1 were incubated with A-LAKs derived from these mouse strains at various E:T ratios at 37°C for 4 h. Each E:T ratio was used for triplicate determinations, and results are shown as mean ± SD. All spontaneous release values were less < 15%.

(B) NZB/BinJ


Day 3
Day 4
Day 5
Day 6
Day 7
% Specific Lysis
0
10
20
30
40
50
60
70
1
10
100
E:T Ratio
RMA/S
RMA/S.CD1.1

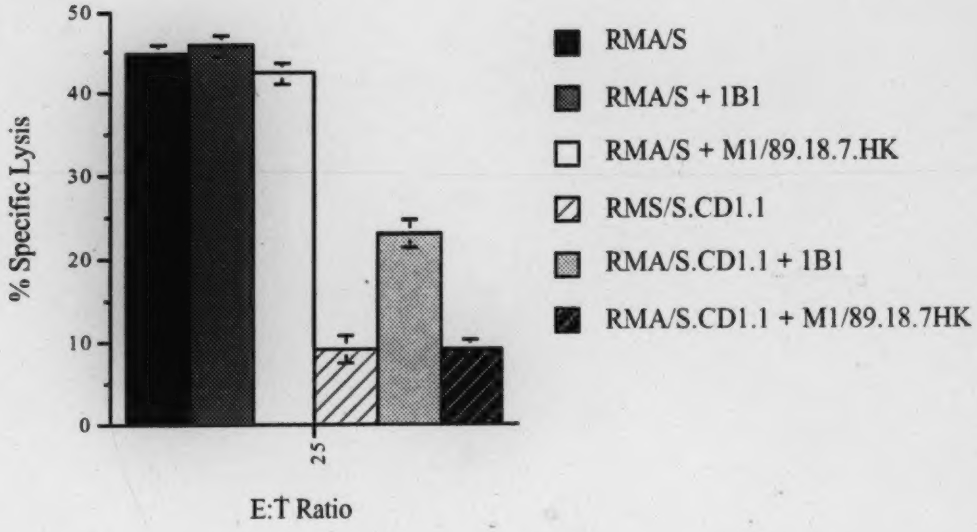


Figure 5-9. The mCD1.1 specific mAb, 1B1, partially restored the lysis of RMA/S.CD1.1 by B6 A-LAK. ^{51}Cr-labeled RMA/S and RMA/S.CD1.1 were preincubated with 1B1 (anti-mCD1) or M1/89.18.7HK (anti-CD45) for 30 min at room temperature. The B6 A-LAKs were then added to each well at an E:T ratio of 25:1, and to give a final mAbs concentration of 20 μg/ml. The cytotoxic assay was then incubated at 37^{o}C for 4 h. Each mAb treatment was carried out in triplicate, and the results are shown as mean ± SD. All spontaneous release values were < 8.6%.

CHAPTER VI

GENERAL DISCUSSION

A. Role of *N*-linked Glycosylation of Class I MHC Molecules in Ly-49 Interaction

With the cloning and characterization of murine NK cell receptors such as Ly-49A and other Ly-49 family members, it is now clear that murine NK cells utilize these classical class I MHC-specific animal C-type lectin receptors to regulate their cytotoxic activities. The identity of Ly-49 receptors to animal C-type lectins led to the suggestion that class I MHC molecule *N*-linked carbohydrate moieties are involved in this particular receptor-ligand interaction. Since there are two conserved *N*-linked and no evidence of *O*-linked glycosylation sites on the $\alpha 1$ and $\alpha 2$ domains of the murine class I MHC molecules, it is only logical to speculate that the carbohydrate moieties on these sites are involved in engaging Ly-49 receptors. Available evidence seems to support this hypothesis and further imply that Ly-49 receptors can recognize specific carbohydrate structures (Daniels et al., 1994b; Brennan et al., 1995). In addition, the CRDs of Ly-49A and C might interact with specific sulfated carbohydrate structures. The speculation that the Ly-49-class I MHC interaction is carbohydrate-dependent is further supported by the fact that the CRD is needed for Ly-49 and class I MHC interaction (Brennan et al., 1996b). As a result, it was a commonly accepted notion that carbohydrate moieties play an important role in this receptor-ligand interaction.

However, conflicting data regarding the involvement of class I MHC *N*-linked carbohydrate moieties in the Ly-49 interaction have recently been provided by the Takei and the Yokoyama groups. By mutating the two conserved *N*-linked glycosylation sites on $\alpha 1$ and $\alpha 2$ domains of H-2D^d, Yokoyama and colleagues found that the expression of the carbohydrate-less D^d can still interact with Ly-49A and protect target cells from lysis by Ly-

49A$^+$ NK cells (Matsumoto et al., 1998). This led them to re-interpret their previous results and propose that the CRD of Ly-49A interacts with some other cell surface carbohydrate structures instead of those on D^d. In other words, they propose that there are two distinct ligands for Ly-49A; class I MHC D^d and an undefined cell surface carbohydrate structure. This is not entirely impossible considering the homodimeric nature of Ly-49 receptors. One can imagine that half of the homodimer interacts with H-2D^d, while the other half of the homodimer simply recognizes an independent carbohydrate ligand. In sharp contrast, using the same mutagenesis strategy, Takei and colleagues found that deletion of the N176 but not the N86 *N*-linked glycosylation site significantly reduces the binding of the mutant D^d-expressing cells to both Ly-49A and Ly-49C transfected COS cells (Lian et al., 1998). In addition, their results also demonstrated that the N176 substitution mutant is Ly-49A$^+$ NK cells sensitive. Therefore, these data strongly suggest the involvement and importance of class I MHC *N*-linked carbohydrate in this receptor-ligand interaction. One can possibly argue that these conflicting data are due to the differences in experimental protocols used. For example, the cell-cell adhesion assays were performed with Ly-49 transfected COS cells in one instance and CHO cells in the other (Lian et al., 1998; Matsumoto et al., 1998). It is possible that other receptor-ligand interactions distinct from the Ly-49-class I MHC interaction could contribute to the binding affinity, particularly perhaps in the case of CHO transfectants where carbohydrate-less class I MHC mediated binding was reported to be observed. Another intriguing observation is the expression of carbohydrate-less H-2D^d in these studies. Since *N*-linked glycosylation is needed for efficient class I MHC assembly, maturation and expression (Shiroishi et al., 1985; Barbosa et al., 1987), the carbohydrate-less D^d might have subtle changes in its conformation. Thus, the different amino acids used by the two groups to substitute the Asn residues in these studies could potentially contribute to these conflicting observations. Nevertheless, the true reasons for these contradicting results remain to be resolved.

To add to the controversy, the crystal structure of the human CD94 homodimer has been determined (Boyington et al., 1999). The structure reveals that the conserved Ca^{2+} binding site found in animal C-type lectins is missing in the CD94 homodimer. Thus, this result shows that NK cell receptors of the animal C-type lectin superfamily adopt a different and novel lectin-fold, and are evolutionary divergent from the conventional animal C-type lectins. Since by definition, C-type lectins generally bind carbohydrates in a Ca^{2+}-dependent manner, this suggests the possibility that NK cell receptors may not normally bind carbohydrates. Furthermore, there has been a suggestion that NK cell receptors are not true animal C-type lectin receptors but rather only lectin-like in their nature, based on the comparison of amino acid sequences of molecules of the greater C-type lectin family (Weis et al., 1998). Because CD94 and the Ly-49 family members bear a certain degree of sequence identity to one another, this raises the possibility that Ly-49 receptors might also adopt structures similar to CD94 homodimers, and potentially incapable of interacting with carbohydrate moieties. Nevertheless, there is strong evidence demonstrating that Ly-49A can indeed bind carbohydrates, as Ly-49A transfected CHO cells have been shown to be able to bind fucoidan (Daniels et al., 1994b). This binding is inhibited by multiple different anti-Ly-49A mAbs, and similarly an interference with Ab binding is observed in the presence of fucoidan (Matsumoto et al., 1998). These data indicate that the CRD of Ly-49 is capable of binding sulfated carbohydrates. Therefore, even though the Ca^{2+} binding site of Ly-49 might not be conserved, it is possible that they bind carbohydrates rather differently from animal C-type lectins and probably in a Ca^{2+}-independent manner. Such Ca^{2+}-independent binding of sulfated carbohydrates has been proposed for P and L-selectins (Varki. 1994). For instance, the binding of sulfatides by selectin seems to take place in a Ca^{2+}-independent binding site. It is possible that similar site(s) also exist on Ly-49 receptors and could account for their abilities to bind anionic polysaccharides.

B. The Importance of Sulfated Carbohydrate Moieties on the H-2D^d and Ly-49A Interaction

As mentioned in Chapter III, no particular satisfying explanation for the conflicting data of Yokoyama and Takei has been available concerning the involvement of class I MHC *N*-linked carbohydrates in Ly-49 interaction. However, our data demonstrating that class I MHC D^d can be sulfated on its *N*-linked carbohydrate moieties and that engagement of D^d requires a threshold Ly-49A density can potentially provide an explanation for these conflicting data. With our *in vitro* cell binding assay, it was demonstrated that the involvement of sulfated carbohydrates in this receptor-ligand interaction is dependent on the Ly-49A density. When the immobilized Ly-49A density is relatively high, the interaction is sulfated carbohydrate-independent. At this end of the Ly-49A density spectrum, our data is in agreement with those obtained by Yokoyama and colleagues (Matsumoto et al., 1998). However, as the density of Ly-49A becomes limiting, the importance of the sulfated carbohydrates in this interaction becomes apparent. In this instance, our data is similar to those of Takei and colleagues (Lian et al., 1998). This implies that sulfated carbohydrate moieties are not an absolute requirement for Ly-49A-D^d interaction, but instead serve to strengthen the affinity or facilitate this particular interaction. In their cell-cell adhesion assays, Yokoyama's group used CHO cells stably transfected with Ly-49A and expressed high levels of this receptor. Whereas, in Takei's studies, the assays utilized COS cells transiently expressing Ly-49A. Although it is difficult to compare the FACS data from these two groups regarding the Ly-49A expression level, COS transfectants generally show a rather heterogeneous expression pattern of this receptor (Takei et al., 1997; Lian et al., 1998). Therefore, it is possible that the *N*-linked carbohydrate-dependent interaction observed by Takei's group is due to a lower level of Ly-49A expression. As a result, the involvement of class I MHC *N*-linked carbohydrate is rather apparent in the cell-cell adhesion system used in their study. Furthermore, since the D^d expression level also influences this receptor-ligand interaction (Kane. 1994), it is possible that different levels of carbohydrate-less D^d might have been expressed by the transfectants used in the studies of Takei and Yokoyama and could also account for the conflicting results. It is possible the Takei transfectants expressed lower levels of carbohydrate-less D^d and were thus less capable of interacting efficiently with Ly-49A. Therefore, when either the Ly-49A or the class I MHC D^d level is low, the presence of *N*-linked carbohydrates on class I MHC may help to stabilize the receptor-ligand interaction.

Conceptually, it is possible to propose a "coordinate interaction" model regarding the recognition of H-$2D^d$ by Ly-49A, based on all of the available data (Fig. 6-1). According to this model, Ly-49A recognition of H-$2D^d$ can be dissected into two distinct phases. The first phase represents the initial contact between Ly-49A and D^d, which is mediated by both *N*-linked carbohydrate moieties and the polypeptide backbone of D^d (Fig. 6-1A and B). The *N*-linked carbohydrate moieties only partially and transiently interact with the Ly-49A CRD, and their major role is to strengthen this initial interaction. As a result, subsequent protein-protein interactions of Ly-49A and D^d can then take place and initiate the inhibitory signals to shut down any potential triggering events. In this second phase of the interaction, the D^d $\alpha 1$ and $\alpha 2$ domains remain associated with Ly-49A, whereas its *N*-linked carbohydrate moieties are no longer needed and might not be necessary to remain associated with the inhibitory receptor (Fig. 6-1C). Nevertheless, the possibility that the *N*-linked carbohydrate moieties remain associated with the CRD during the second phase of the interaction cannot be ruled out (Fig. 6-1D). The advantage of this model is it does not require the Ly-49 receptor to recognize other ligands besides class I MHC as proposed by Yokoyama and colleagues (Matsumoto et al., 1998). This is especially important since no other ligands, besides class I MHC, have been identified for Ly-49 receptors. Furthermore, the proposal of a quantitative and not necessarily qualitatively contribution of *N*-linked carbohydrates certainly provides an explanation for the conflicting results of Yokoyama and Takei.

It is noteworthy that according to this model, the class I MHC *N*-linked carbohydrate moieties involved in Ly-49 interaction might not need to be sulfated at all. However, it can be postulated that in the presence of sulfated carbohydrate moieties, the affinity of the initial contact is further increased compare to the non-sulfated class I MHC molecules. This hypothesis not only integrates our data into the model, but also the observation that Ly-49A CRD can bind sulfated-fucose containing polysaccharides. Consistent with this idea, the T lymphoma NZB1.1, which expresses lower levels of D^d compared to S49.1 and A20.1, is able to bind to isolated Ly-49A equally well, if not better, than those cell lines expressing high levels of D^d. It is possible that the highly sulfated carbohydrates on NZB1.1 D^d indeed help to increase their D^d interaction affinity with Ly-49A. As a result, even with lower class I MHC D^d expression, binding to isolated Ly-49 is still possible.

Using the Ly-49A and D^d interaction as an example, this model predicts Ly-49A recognizes H-2D^d subsets in a hierarchical manner. Assuming constant Ly-49 and H-2D^d densities, this model predicts that Ly-49A prefers to interact with D^d containing sulfated carbohydrates, then possibly D^d without this post-translational modification, and finally the carbohydrate-less D^d (Fig. 6-2). Since *N*-linked carbohydrates are required for efficient class I MHC expression on the cell surface (Shiroishi et al., 1985; Barbosa et al., 1987), engagement of Ly-49 receptors with carbohydrate-less class I MHC molecules on the cell surface is rather unlikely *in vivo*. The prediction of a hierarchical Ly-49A-D^d interaction creates another level of complexity in this receptor-ligand interaction. For instance, do different D^d glycoforms interact with Ly-49A with equal efficiency? What is the consequence of the varying degree of *N*-linked glycosylation on this receptor-ligand interaction? These are the questions that remain to be answered.

Extent and Type of Class I MHC Glycosylation and Ly-49A Interaction

It is known that significant heterogeneity of charge and size of class I MHC molecules is contributed by the microheterogeneity of *N*-linked glycosylation (Sweidler et al., 1983; Krakauer et al., 1980; Landolfi and Cook. 1986). Our data obtained by immunoprecipitation of D^d from different tumor cell lines demonstrated the same point. If the role of class I MHC *N*-linked carbohydrates is to strengthen its interaction with Ly-49A, then the specific glycoforms of D^d might aid this process with different efficiencies. Therefore, the outcome of Ly-49A engagement with D^d might not be as predicted from the level of D^d expression, even in the absence of sulfation. For example, let us assume that there are two target cells expressing the same low level of D^d that may be just sufficient to initiate inhibitory signals through Ly-49A. However, only one of them expresses D^d glycoforms that is able to engage this inhibitory receptor efficiently. In this instance, lysis will be detected in one target but not the other, even though both target cells are expressing equally low levels of D^d. To further complicate the issue, the process of sulfation can potentially change the dynamics of this interaction. Using the same example, the target cells expressing D^d glycoforms that fail to engage Ly-49A may be modulated to do so by simply adding sulfate groups to its carbohydrate moieties. Both target cells would now be resistant to Ly-49A$^+$ NK cell lysis. Thus, another dimension can be added to the proposed hierarchical Ly-49A and D^d interaction.

Obviously, the model proposed in the previous section and this section are not restricted to Ly-49A, but also may be applicable to other Ly-49 family member interactions with their respective class I MHC ligands. This is not an unreasonbale assumption, since Ly-49C also failed to interact with carbohydrate-less D^d (Lian et al., 1998). Therefore, both the extent and type of complex *N*-linked glycosylation and sulfation of class I MHC could constitute another regulatory mechanism to modulate these NK cell receptor-ligand interactions.

N-linked Sulfated Carbohydrates and the Receptor Calibration Model

The receptor calibration model operates on the basis that the total avidity of the inhibitory interaction is one of the deciding factors determining NK cell cytotoxic activity. Therefore, the proposal that class I MHC *N*-linked carbohydrates can influence the affinity of class I MHC interactions with inhibitory receptors has immediate consequences on the receptor calibration model. Since *N*-linked carbohydrate modification of class I MHC is heterogeneous, this means Ly-49 receptors have to be calibrated to a level that will be sensitive to a reduction in one or more class I MHC glycoforms. As a result, the avidity contributed by each class I MHC glycoform during the calibration process may be critical. For instance, it is proposed that Ly-49A in H-2^d mice is calibrated to a level so that total inhibitory signals received by the Ly-$49A^+$ NK cells through D^d is just a little above the minimum threshold required to override the triggering events. Thus, a small reduction of D^d level is sufficient to induce target cell lysis (Höglund et al., 1997).

According to the coordinate interaction model, this minimum threshold should be attained more readily in the presence of sulfated carbohydrate moieties even with a low density of D^d. A similar argument might be made for other class I MHC carbohydrate modifications that may influence Ly-49 interaction, since resting and activated cells can express different class I MHC glycoforms (Landolfi and Cook. 1986). This supports the notion that the Ly-49 receptor calibration of mature NK cells has to be an ongoing process.

C. Involvement of Sulfated Carbohydrate on H-$2D^d$ and Its Interaction with Other Ly-49 Molecules

H-$2D^d$ has been demonstrated to be a cognate ligand for multiple Ly-49 receptors including Ly-49A, C, G2 and D (Kane. 1994; Mason et al., 1995; Lian et al., 1998; Mason et al., 1998). This simply reflects the observation that each Ly-49 receptor can interact with a spectrum of class I MHC allelic products (Takei et al., 1997). These observations raise a fundamental question; why has evolution seemingly created and maintained such a redundant system? To reduce the complexity of the NK cell regulatory system, would it not be an evolutionary advantage to have inhibitory or activating receptors that recognize a relative conserved region of class I MHC, such as the $\alpha 3$ domain? One major disadvantage of a single regulatory receptor is the inflexibility that will be imposed within the system, where the loss or reduction in expression of a single class I MHC allelic product may go undetected. To be able to detect the loss of a single class I MHC allelic product may be particularly useful to combat viruses that may target expression of individual class I MHC alleles that may be responsible for presentation of peptide antigens derived from that virus. In addition, expression of a single NK cell regulatory receptor will constrain the missing self to become an "all or none" phenomenon. In other words, there will be an absolute threshold that class I MHC has to be reduced to, before the inhibitory signals can be relieved. However, in this hypothetical scenario, if only one class I MHC allelic product is down regulated, the target cells would in all likelihood still remain resistant to NK cell lysis. Under these circumstances, expression of a single class I MHC-specific NK cell receptor by all NK cells will render them completely useless. Therefore, from a functional viewpoint, the existence of multiple NK cell receptors recognizing a broad spectrum of class I MHC definitely offers certain advantages.

Although Ly-49 receptors are capable of interacting with multiple class I MHC allelic products, it is unlikely that they bind each class I MHC with identical affinities and are each likely to recognize class I MHC ligands in a hierarchical manner. Even though H-2D^d is known to bind to multiple Ly-49 receptors in *in vitro* assays, but it might not be the major ligand for most NK cell receptors. For example, it can be argued that under normal physiological conditions, H-2D^d is not the major ligand for a receptor such as Ly-49G2. Several lines of evidence have suggested that the affinity of this particular interaction is relatively low. These include the findings that introduction of the $\alpha 1$ and $\alpha 2$ domains of D^d in the form of a chimeric class I MHC gene in H-2^b mice does not down regulate Ly-

49G2 expression level as dramatically as seen with Ly-49A (Salcedo et al., 1997; Johansson et al., 1998). In addition, our data on the reduction of Ly-49G2 expression in 129/J mice suggests that this inhibitory receptor may indeed recognize class I MHC molecules other than D^d. Furthermore, *in vitro* cell-cell adhesion assays also failed to detect binding of H-$2D^d$-expressing cells to Ly-49G2 (Takei et al., 1997).

Since resting splenocytes do not seem to express detectable sulfated glycoforms of D^d, this might mean that D^d has a low affinity interaction with Ly-49G2 and thus cannot downwardly calibrate NK cell Ly-49G2 receptor expression. Nevertheless, it has been demonstrated that Ly-49G2$^+$ NK cell cytotoxic activity is inhibited by H-2^d Con A blasts (Mason et al, 1995). Since a small subset of D^d is sulfated upon Con A activation of T cells (Chapter III), this structural change may increase the affinity of their D^d interaction with Ly-49G2. It is important to note that other changes in *N*-linked carbohydrate composition could possibly serve the same purpose. It is known that upon Con A activation, the class I MHC *N*-linked carbohydrate composition is different from that of resting T cells (Landolfi and Cook. 1986). However, whether changes in carbohydrate composition distinct from sulfation are sufficient to influence the affinity of the receptor-ligand interaction cannot be ruled out.

These possibilities can certainly be extended to other Ly-49 receptors in their interaction with class I MHC ligands. Therefore, even though *N*-linked carbohydrate might not determine the specificity of the inhibitory receptors, they may still influence the hierarchy of class I MHC alleles with which specific Ly-49 receptors interact.

Hypothetical Functions of Activating NK Cell Receptors

The existence of class I MHC-specific activating receptors, either from Ly-49 or CD94/NKG2 families, are not a predictable outcome of the missing self hypothesis (Ljunggren and Kärre. 1990). However, they do not necessarily present a conflict to the missing self hypothesis and the receptor calibration model. These activating molecules can be viewed as contributing to triggering events and constituting part of the threshold that the inhibitory signals have to overcome in order to prevent target cell lysis. Because Ly-49D also interacts with a panel of class I MHC molecules including D^d (Nakamura et al., 1999; Thaddeus et al., 1999), the $Ly49A^+D^+$ NK cells will thus receive both activation and inhibitory signals through the same ligand. For this particular NK cell population, the strength of the signals generated through these two receptors is an important deciding factor in determining the lytic activity of this NK cell subset. If Ly-49A and D interact with various D^d glycoforms in a different hierarchical manner, then the subsets of D^d being expressed will play a role in balancing activation and inhibitory signals. For instance, if Ly-49A interacts with most of the D^d glycoforms on the cell surface and/or more efficiently than Ly-49D, an inhibitory outcome will be observed. Therefore, depending on which class I MHC glycoforms are expressed, the NK cell sensitivity of a particular target might not be predictable from the expression level of class I MHC.

This speculative differential Ly-49A and D recognition of D^d glycoforms could, in part, account for the conflicting data obtained by Takei and Yokoyama. Due to the overlapping expression pattern of Ly-49 receptors, at least some of NK cells used by both research groups would be Ly-$49A^+D^+$. If Ly-49D interacts with carbohydrate-less D^d better than Ly-49A, the activation state of Ly-$49A^+D^+$ NK cells will thus be higher, and would require a stronger inhibitory signal to shut down the NK cell cytotoxic function.

D. Sulfation of Class I MHC *N*-linked Carbohydrates and Immune Surveillance

An "immune surveillance" theory was proposed in the early part of this century. Since then multiple modifications have been added to the original proposal. However, the fundamental concept of the theory is that malignant or transformed cells that arise in the body are constantly kept under control by the immune system. Due to their cytotoxic activities against tumor cells, both NK cells and CTLs are implicated in this immune surveillance. Even before the cloning of any NK cell regulatory receptors, existing evidence has suggested that glycosylation on target cells plays an important role in modulating NK cell cytotoxicity. For instance, the murine tumor cell line MDAY-D2 can be rendered NK cell resistant simply by treating them with wheat germ agglutinin (WGA), a lectin that binds sialic acid, and selecting the WGA resistant variants (Nestel et al., 1984; Takano et al., 1994). Aggressive and metastatic tumors tend to have altered glycosylation patterns (Dennis et al., 1987; Fukuda. 1985; Saitoh et al., 1992). These observations indicate the fact that changes in the glycosylation process can have important consequences upon tumor survival and possibly their susceptibility to immune surveillance. With the realization that NK cells are regulated directly by class I MHC through C-type lectin-like receptors, changes in the glycosylation pattern of tumor cell class I MHC might be a major mechanism for tumor cells to evade NK cell surveillance.

As presented in chapter III, sulfated H-2D^d is readily observed in tumor cell lines. If *N*-linked glycosylation on class I MHC indeed modulates NK cell cytotoxic activity, then what are the advantages of class I MHC sulfation in tumor cell survival? What are the immune selection pressures being exerted on the tumor cells in order to favor such post-translational modification? The answer probably comes from the fact that in order to evade immune surveillance, tumor cells have to deal with both the innate and adaptive immune responses. As mentioned above, NK cell cytolytic functions may be affected directly by changes in class I MHC glycosylation patterns. Since TCRs do not recognize the *N*-linked carbohydrate on class I MHC molecules (Goldstein and Mescher. 1985; Miyazaki et al., 1986), the effect of this post-translational modification on CTL killing is not entirely clear. Nevertheless, recent evidence has shown that differential *N*-linked glycosylation on class I MHC K^b does have important consequences on T cell functions, as a heavily glycosylated K^b subset failed to trigger allo-specific T cell adhesion and response (Shen and Kane. 1995). Thus, it is possible that changes in class I MHC glycosylation patterns are also sufficient for tumors to escape CTL responses. However, reduction or loss of class I

MHC expression remains one of the major mechanisms for tumors to evade T cell immunity (Tanaka et al., 1989; Garrido et al., 1995). Nevertheless, a complete down regulation of class I MHC expression is obviously undesirable and will render tumor cells sensitive to NK cell lysis. For instance, tumor cells might down regulate their class I MHC expression level to the point that it no longer elicits an effective CTL response, but is insufficient to adequately engage NK cell class I MHC-specific inhibitory receptors, and thus be sensitive to NK cell lysis. Thus, the extent of class I MHC down regulation is a delicate balance for tumors to evade the constant challenges from both the innate and adaptive immune responses. To circumvent this problem, sulfation of class I MHC *N*-linked carbohydrates could offer a much needed solution. Based on the coordinate interaction model, sulfated class I MHC molecules could potentially engage NK cell inhibitory receptors such as Ly-49A more efficiently than their non-sulfated counterparts. As a result, even a low level of class I MHC expression would possibly still be able to protect a tumor cell from NK cell lysis, and meanwhile, due to low class I MHC expression, not induce a CTL response.

If NK cell inhibitory receptors do recognize class I MHC in a hierarchical manner as proposed in previous sections, sulfation of class I MHC might yet offer another level of protection for tumors from NK cell lysis. The process of sulfation might enable class I MHC molecules to interact with other Ly-49 receptors that they normally do not engage or engage with low affinity. For instance, sulfated D^d could potentially interact with Ly-49C, G2 or other Ly-49 inhibitory receptors with greater affinity than it normally would. Consequently, the cytotoxic activity of a larger population of NK cells will be inhibited even by the reduced level of class I MHC molecules that may be expressed by some tumor cells. In addition, if NK cell activating receptors such as Ly-49D interact with sulfated class I MHC molecules with lower affinity, the signals generated will be less likely to activate NK cells and spare tumor cells from lysis. It is to be noted that the above hypothesis does not dismiss the importance of changes in the glycosylation pattern of the tumor cells in evading immune surveillance, but simply adds another level of complexity to it.

The original tumor immune surveillance theory was specifically formulated to explain how the immune system meets challenges by tumors and transform cells. However, similar arguments for class I MHC sulfation as a mechanism to evade NK cells immunity can also be made for viruses and other pathogens. To this end, there is no available evidence demonstrating that viruses actually encode sulfotransferase genes in their limited genetic materials. Nevertheless, it remains possible that hypersulfation of class I MHC can be achieved indirectly by up regulating specific host sulfotransferase activities or PAPS synthesis or translocation. Therefore, for those viruses that down regulate host class I MHC expression, induction of class I MHC sulfation may provide a distinct advantage in evading host immune responses due to effects on NK cell regulation.

E. Regulation of NK Cell Cytotoxic Activities by Non-MHC Linked Nonclassical Class I-Like Molecules.

It is clear that multiple NK cell receptor families are utilized to interact with both classical and nonclassical class I MHC molecules. Available evidence suggests that evolution has adopted different receptors to interact with both classical and nonclassical class I MHC molecules. For instance, the CD94/NKG2 receptor family is demonstrated to interact with the nonclassical class I MHC (Braud et al., 1998; Borrego et al., 1998). Therefore, it is not altogether surprising that mCD1.1 has the ability to modulate NK cell cytotoxic activity.

Our data suggests the existence of a conserved mCD1.1-specific NK cell inhibitory receptor(s). Even though the nature of this receptor(s) remains undefined at this moment, it is not difficult to reason the need for such an inhibitory receptor(s). For instance, Stenger and colleagues recently demonstrated that infection of human antigen presenting cells with *Mycobacterium tuberculosis* resulted in down regulation of CD1 expression (Stenger et al., 1998). Since CD1 molecules are capable of presenting *M. tuberculosis* glycolipids to CD1-specific T cells (Jullien et al., 1997), they proposed that down regulation of CD1 levels serves as an escape mechanism for this pathogen to avoid immune recognition (Stenger et al., 1998). As a result, a reservoir of *M. tuberculosis* is established and persistent infection becomes possible. However, more importantly, the expression of other antigen presenting molecules including class I and II MHC are not affected. Under this circumstance, existence of an NK cell expressed CD1-specific inhibitory receptor(s) would be beneficial. For those NK cells expressing the putative CD1-specific inhibitory receptor(s), the inhibition mediated by CD1 is now no longer present and lysis of the pathogen infected APCs may proceed (Fig. 6-3). However, the unaltered classical class I MHC expression does impose certain restriction on the lysis of *M. tuberculosis* infected APCs. For instance, if the NK cell subsets express both class I MHC and CD1-specific inhibitory receptors, then the inhibitory signals generated by the class I MHC-specific receptor are a critical issue. Nevertheless, based on the receptor calibration model, these NK cells would be calibrated on all of these self-inhibitory receptors. Therefore, with the reduction of CD1 expression alone, it may be sufficient to relieve the inhibitory signals and allow the triggering events to take over.

The above proposal obviously would not be restricted to *M. tuberculosis* infection. Similar scenarios can be extended to other mycobacterium infection or pathogens that down regulate CD1 expression but not the classical class I MHC expression.

Putative mCD1.1-Specific Inhibitory Receptor and NKT Cells

Our study on the inhibitory effects of mCD1.1 expression is mainly focused on the regulation of NK cell cytotoxic activity. However, it is also possible that the putative mCD1.1-specific inhibitory receptor(s) has a broad cellular distribution that includes NKT cells. Since the nature of this receptor(s) is not known, this hypothesis cannot be tested at the present time. Nevertheless, this is not an unreasonable assumption since NK cells and

NKT cells share the same lineage (Carlyle et al., 1998). Furthermore, expression of various inhibitory receptors is not restricted to NK cells, as in addition to NK cells, the Ly-49 inhibitory receptors are also expressed by subsets of $CD3^{+}NK1.1^{+}$ NKT and $CD3^{+}NK1.1^{-}$ T cells (Ortaldo et al., 1998). Upon engagement with class I MHC, these inhibitory receptors can down regulate cytokine production and the cytotoxic activity of these T cells (Ortaldo et al., 1998). Similar results have been demonstrated with human CTLs as crosslinking of the p58 KIR on CTLs can partially inhibit their cytotoxic activities (Ferrini et al., 1994).

If NKT cells do express the putative CD1-specific inhibitory receptor(s), what is the advantage of expressing such receptor(s)? The answer may lie in the observation that the pool of mCD1.1 autoreactive T cells can be quite large. It is estimated that these T cells range from 0.5 to 2% of the lymphocytes in the spleen, and comprise a much larger percentage of the T cells in the bone marrow and liver (Bendelac et al., 1997). However, it is not entirely clear how these autoreactive T cells are kept from being activated under normal circumstances. Bendelac and colleagues have suggested that the autoreactivity of these T cells is normally suppressed by MHC-specific inhibitory receptors (Park et al., 1998). They further suggested that loss of negative regulation through these MHC-specific inhibitor receptors could lead to activation of these autoreactive T cells (Park et al., 1998). Consistent with this view, the mCD1 autoreactivity NKT cells is most striking when T cell hybridomas made from this population are analyzed. These hybridomas tend to lose expression of NK1.1, and as a result of fusion, they may lose expression of other NK cell inhibitory receptors and thereby reveal their autoreactive nature. Therefore, it is possible that the mCD1.1-specific inhibitory receptor(s) suggested by our study may also be originally expressed by these mCD1.1-specific autoreactive T cells. The suggestion that the NKT cell autoreactivity is inhibited by a mCD1.1-specific inhibitory receptor(s) offers a distinct advantages over specific inhibition mediated by self-classical class I MHC molecules. Based on the random distribution of NK cell inhibitory receptors, it is unlikely that all autoreactive NKT cells will co-express a self-classical class I MHC-specific inhibitory receptor. As a result, their autoreactivities might not be inhibited by classical class I MHC. However, with the proposal that NKT cells express the putative mCD1-specific inhibitory receptor(s), the inhibition will be directly mediated by mCD1.

Self-Glycosylphosphatidylinositol and mCD1.1-Specific Inhibitory Receptor(s)

Current opinions regarding mouse NK cell inhibitory receptor engagement with class I MHC molecules indicate that it is independent of the specific antigen being presented in the antigen binding groove. This conclusion is based on studies of the best characterized Ly-49 family member, Ly-49A. Its interaction with D^d appears to be peptide dependent, but not peptide specific (Correa et al., 1995). Therefore, as long as D^d is stabilized by peptide antigens, inhibitory signals can still be initiated and shut down Ly-49A$^+$ NK cell cytotoxic activity (Correa and Raulet. 1995; Orihuela et al., 1996). However, recent data has shown that Ly-49C can discriminate between different forms of K^b on the cell surface (Su et al., 1998). Furthermore, the CD94/NKG2A heterodimer only recognizes HLA-E in the presence of leader peptides derived from other HLA products (Braud et al., 1998; Borrego et al., 1998). Therefore, the antigen dependent, but not antigen specific, recognition of D^d by Ly-49A might not be generalized to other NK cell regulatory receptors.

Assuming the existence of a mCD1.1-specific inhibitory receptor(s), it is possible mCD1.1 can regulate NK cell or NKT cell functions in a ligand-dependent manner. Although CD1 molecules are capable of binding and presenting non-self antigenic glycolipids to NKT cells (Kawano et al., 1997; Burdin et al., 1998), mass spectrometry and radiolabeling experiments have directly demonstrated that the majority of the material bound to mCD1.1 in normal cells is a self-glycosylphosphatidylinositol (Joyce et al., 1998). Therefore, it is possible that the mCD1.1-specific receptor(s) may represent a means by which autoreactive NKT cell functions can be specifically inhibited. For instance, perhaps the engagement of mCD1-specific inhibitory receptor(s) on autoreactive NKT cells only becomes possible when mCD1 is bound with a self-ligand (Fig. 6-4A). In this scenario, expression of mCD1.1-specific inhibitory receptor(s) by non-autoreactive NKT cell will not be a concern. If mCD1.1 presents a non-self glycolipid recognized by the NKT TCR, but not by the mCD1.1-specific inhibitory receptors, the normal function of the NKT cell is not affected (Fig. 6-4A). Therefore, the suggestion that this putative inhibitory receptor(s) recognizes mCD1.1 in a ligand-dependent manner is certainly an attractive model for suppressing the mCD1.1-specific autoreactive NKT cells. In addition, a similar ligand-dependent inhibitory effect of CD1 can be extended to NK cells. For example, for those pathogens that do not down regulate CD1 expression to significant level, but may provide glycolipid antigens to substitute for endogenous self-glycolipid bound in CD1, this might still enable NK cells to specifically lyse infected CD1$^+$ target cells (Fig. 6-4B).

Since the antigens presented by CD1 in part consist of carbohydrate components, it is interesting to speculate whether the putative mCD1.1 inhibitory receptor(s) also recognizes carbohydrates as suggested for Ly-49 receptors. Therefore, if the putative mCD1.1-specific inhibitory receptor(s) recognizes the $\alpha 1$ and $\alpha 2$ domains of mCD1.1, the carbohydrate moieties of the antigen presented by mCD1.1 could play a role in this receptor-ligand interaction. Interestingly, mCD1.1 possesses five *N*-linked glycosylation sites on its $\alpha 1$ and $\alpha 2$ domains (Bradbury et al., 1988). It is possible that these carbohydrate components can strengthen mCD1 interaction with the putative mCD1.1-specific inhibitory receptor(s) as discussed in previous sections. Nevertheless, these hypotheses can only be verified when the nature of the putative mCD1-inhibitory receptor(s) has been identified.

F. Self and Non-Self Discrimination of NK Cells

The discovery of NK cell regulatory receptors seems to provide a basic mechanism for NK cells to discriminate self from non-self. It is clear that unlike B and T cells, NK cells achieve this goal by monitoring the class I MHC expression levels through class I MHC-specific receptors (Ljunggren and Kärre. 1990). At first glance, it seems that the self and non-self discrimination for NK cells can be defined over a broad spectrum of class I MHC levels, and might not be as strictly defined as in B and T cells. However, with the prediction of the receptor calibration model, the margin for self definition for NK cells may be considerably narrowed (Olsson et al., 1995). The data presented in chapter II demonstrating a threshold and sharp receptor density dependence of inhibitory receptor engagement certainly agree with the idea of a narrow window of self definition. In addition, the data presented in chapter III suggest that *N*-linked glycosylation could be a factor in determining the range of this possibly narrow margin of self and non-self determination. This is especially important since, unlike TCRs, inhibitory receptors do not seem to discriminate the antigen being presented by class I MHC molecules. As a result, this allows greater flexibility within the system, so that the absolute level of class I MHC expression will not be the sole factor that determines what is self and what is non-self.

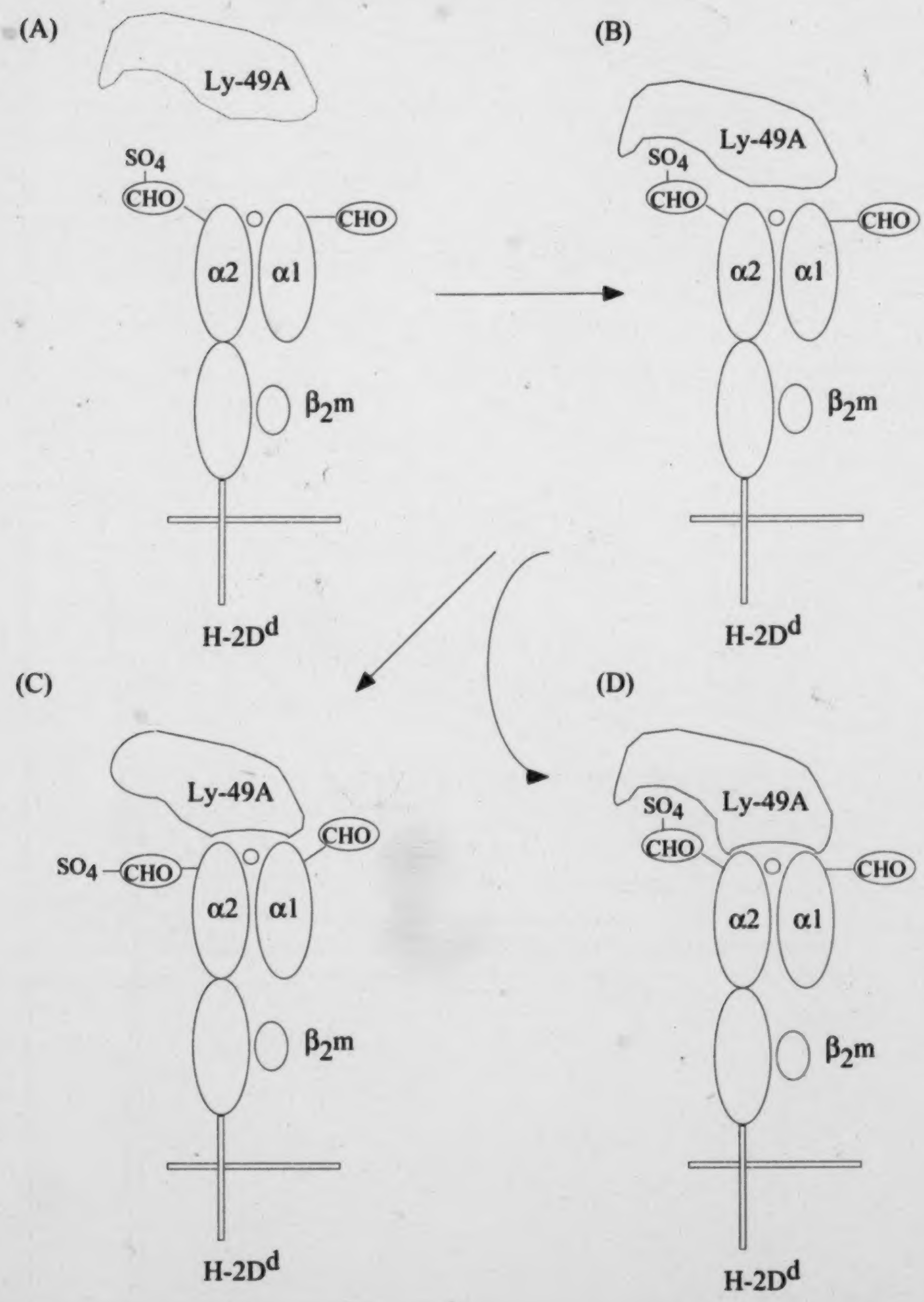
G. Future Directions

Our understanding of NK cell functions has come a long way since the discovery of NK cells in 1975. Nevertheless, several fundamental issues and conflicting data remain to be resolved. For instance, the precise roles of class I MHC *N*-linked carbohydrates on Ly-49 receptors have to be analyzed in more detail. Through the BIAcore system, it should be possible to measure the affinity of the Ly-49A and D^d interaction, and the effect of sulfated carbohydrates on the affinity of this receptor-ligand interaction. Furthermore, this experimental system should reveal whether Ly-49A preferentially interacts with certain D^d

glycoforms. The other issue that needs to be addressed is the ligand specificities of other Ly-49 receptors, such as Ly-49B, E and F. For these receptors, mAbs specific for them first need to be developed. However, for Ly-49G2, the two new mAbs should make the detailed analysis of Ly-49G2 specificity and function a possibility and resolve some of the reported conflicting results. Lastly, the identity of the putative mCD1.1-inhibitory receptor(s) needs to be defined. This receptor could play an important role in our understanding of the fundamental biology of both NK and NKT cells.

Figure 6-1. Schematic diagram representation of the coordinate interaction model. This model proposes that there are two phases of Ly-49A-D^d interaction. During the initial contact, Ly-49A first interacts with the $\alpha 1$ and $\alpha 2$ domains of D^d (A and B). In addition to this protein-protein interaction, Ly-49A also interacts with the carbohydrate structures on the N176 glycosylation site of D^d (B). This model suggests that this protein-carbohydrate interaction may help to strengthen the affinity of this initial contact and the overall receptor-ligand interaction. As a result, the interaction can proceed through the second phase, which is simply mediated by protein-protein interactions (C). The other possibility is that the CRD domain of Ly-49 remains associated with the *N*-linked carbohydrates on residue N176 (D). As a result, stronger inhibitory signals can be generated due to more Ly-49 receptors engaged. Nevertheless, based on this model, the involvement of class I *N*-linked carbohydrates in the initial phase of interaction is not an absolute requirement.

Coordinate Interaction Model


(A)
Ly-49A
SO4
CHO
CHO
α2
α1
β2m
H-2Dd
(B)
Ly-49A
SO4
CHO
CHO
α2
α1
β2m
H-2Dd
(C)
Ly-49A
CHO
SO4
CHO
α2
α1
β2m
H-2Dd
(D)
SO4
Ly-49A
CHO
CHO
α2
α1
β2m
H-2Dd

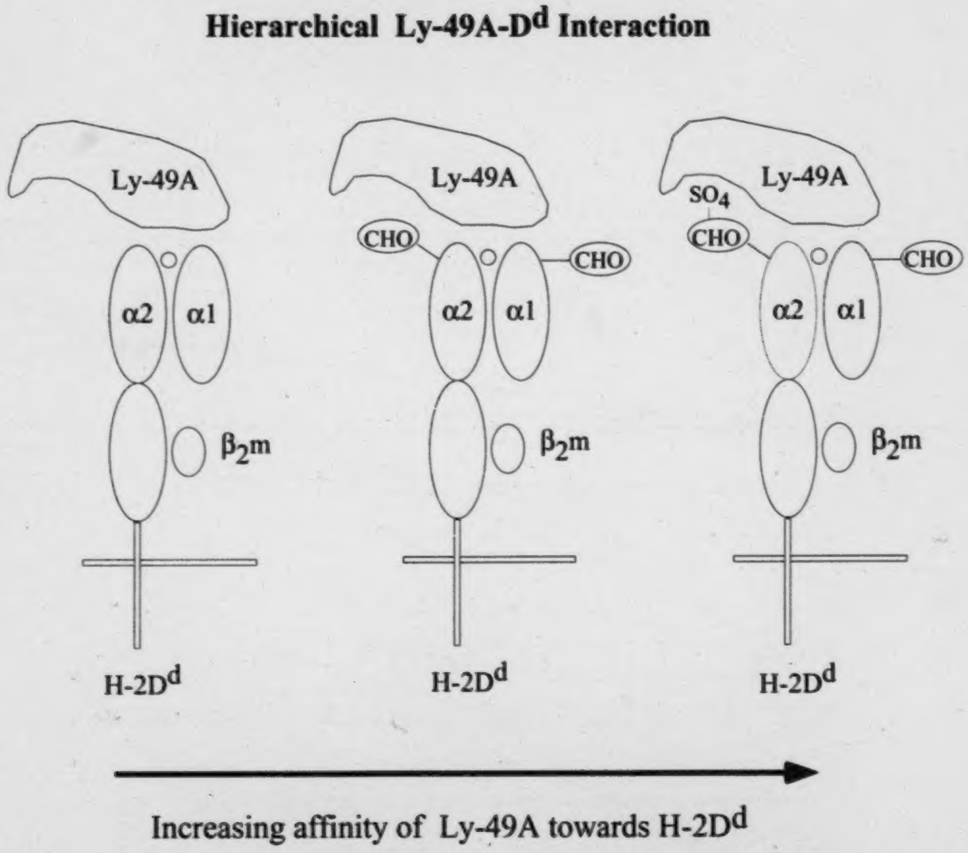

Hierarchical Ly-49A-D^d Interaction
Ly-49A
α2
α1
β$_2$m
H-2D^d
Ly-49A
CHO
CHO
α2
α1
β$_2$m
H-2D^d
SO$_4$
Ly-49A
CHO
CHO
α2
α1
β$_2$m
H-2D^d
Increasing affinity of Ly-49A towards H-2D^d

Figure 6-2. Ly-49A interacts with H-2D^d in a hierarchical manner. Since the coordinate interaction model proposed in figure 6-1 suggests that *N*-linked carbohydrates on H-2D^d can strengthen class I MHC interaction with Ly-49A receptor, this further implies that Ly-49A might interact with different H-2D^d subsets in a hierarchical manner. For instance, Ly-49A might preferentially interact with D^d with sulfated *N*-linked carbohydrates, this is then followed by those without sulfate groups, and finally those without any *N*-linked carbohydrates.

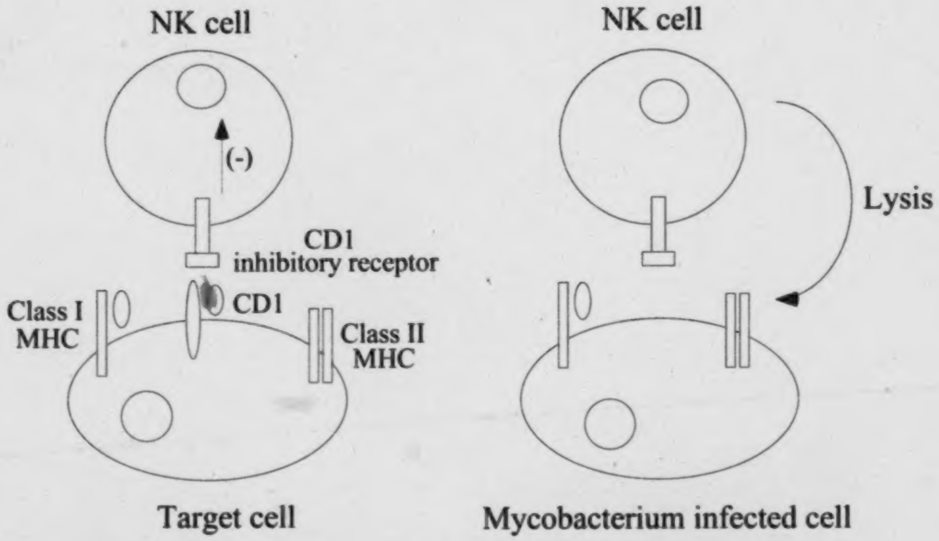
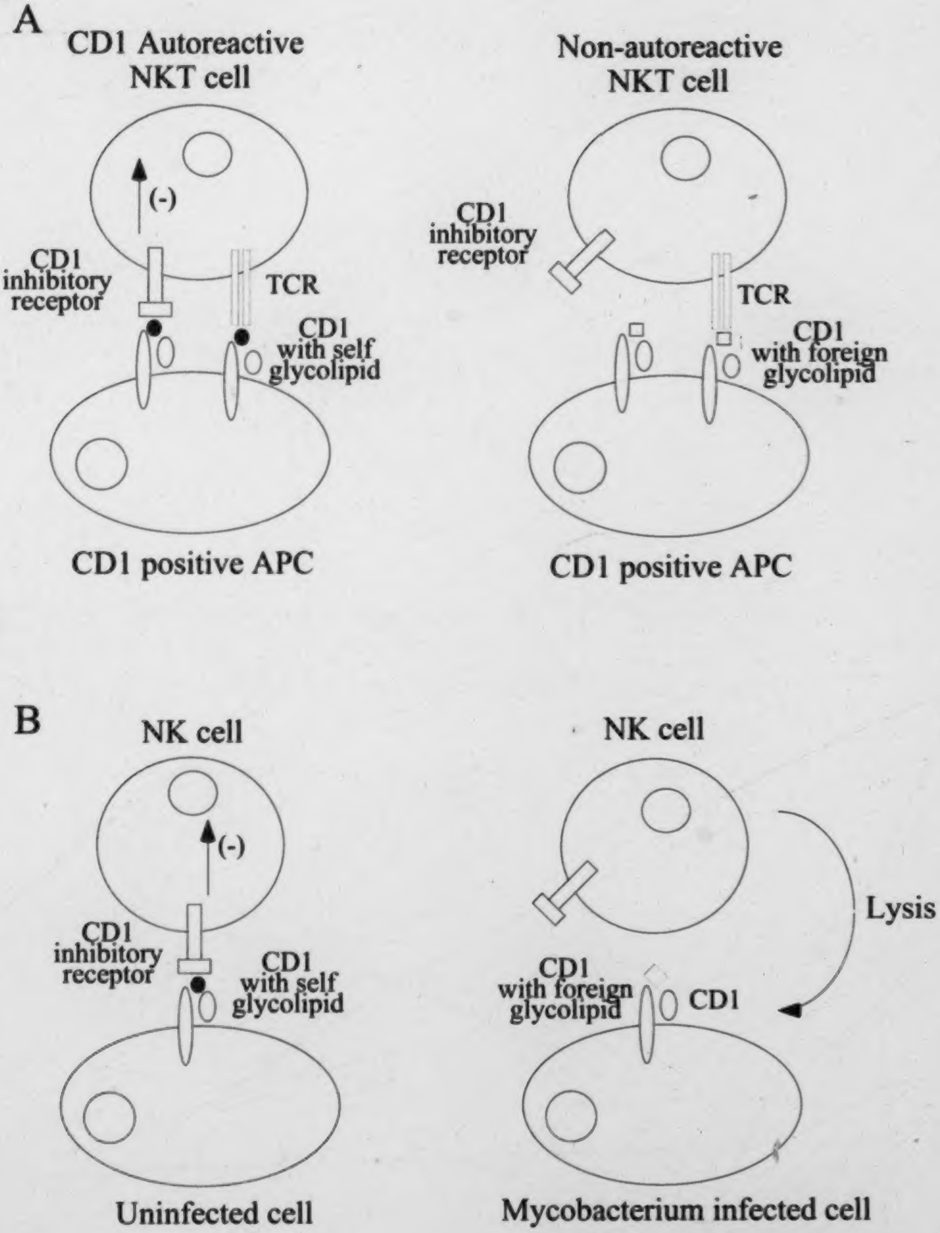

NK cell
NK cell
(-)
Lysis
CD1
inhibitory receptor
Class I
MHC
CD1
Class II
MHC
Target cell
Mycobacterium infected cell

Figure 6-3. Models of the CD1 regulation of NK cell function. The putative CD1-specific inhibitory receptor(s) might enable NK cells to lyse target cells that down regulate CD1 expression. Under normal physiological conditions, CD1 expression would inhibit NK cell cytotoxic activity. However, during *M. tuberculosis* infection, CD1, but not classical class I MHC, expression is down regulated. As a result, NK cells bearing the CD1-specific inhibitory receptor(s) is no longer inhibited, and lysis of the mycobacterium infected cell will take place.

Figure 6-4. Regulation of autoreactive NKT cells and NK cells by mCD1.1-specific inhibitory receptor(s) in an antigen-dependent manner. (A). In this model, both autoreactive and non-autoreactive NKT cells express the CD1-specific inhibitory receptor(s). For autoreactive NKT cells, the autoreactivity is suppressed in the presence of self-glycolipid presented by mCD1.1. Whereas, for non-autoreactive NKT cells which recognize foreign glycolipids in the context of mCD1.1, its normal function is not inhibited by the mCD1.1 self glycolipid-specific inhibitory receptor(s). (B). It is possible that the putative mCD1.1-inhibitory receptor(s) also regulates NK cells in a similar ligand-dependent manner. Under normal circumstances, presentation of a self-glycolipid by mCD1.1 will result in inhibition of NK cell cytotoxic activity. However, when bacterial glycolipids are loaded onto mCD1.1, inhibition mediated through mCD1.1-specific inhibitory receptor(s) is no longer possible. As a result, lysis of the target cell in the absence of significant mCD1.1 down regulation can still take place.


A
CD1 Autoreactive
NKT cell
(-)
CD1
inhibitory
receptor
TCR
CD1
with self
glycolipid
CD1 positive APC
Non-autoreactive
NKT cell
CD1
inhibitory
receptor
TCR
CD1
with foreign
glycolipid
CD1 positive APC
B
NK cell
(-)
CD1
inhibitory
receptor
CD1
with self
glycolipid
Uninfected cell
NK cell
Lysis
CD1
with foreign
glycolipid
CD1
Mycobacterium infected cell
(No CD1 down regulation)

CHAPTER VII

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